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13. ABSTRACT *(Maximum 200 Words)*

Excessive consumption of dietary fat may enhance the rate of breast cancer metastasis. In addition, it is generally accepted that the upregulation of endothelial cell adhesion molecules is involved in the formation of blood-borne metastasis. Among different adhesion molecules, evidence indicates that intracellular adhesion molecule-1 (ICAM-1) may play a critical role in breast cancer metastatic formation. Our studies have demonstrated that dietary fatty acids can exert highly specific effects on NF-κB activation and expression of adhesion molecules in human endothelial cells. In addition, we indicated that linoleic acid induces ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1) expression through the activation of NF-κB. More importantly, because the ability of cancer cells to interact with the endothelium appears to be a prerequisite for the potential of distant metastasis and because ICAM-1 and VCAM-1 are considered to be a crucial adhesion molecule in this process, the present study may have significant therapeutic implications in developing a novel strategy against cancer metastasis.

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Table of Contents

1. INTRODUCTION

Excessive consumption of dietary fat may enhance the rate of breast cancer metastasis. In addition, it is generally accepted that the upregulation of endothelial cell adhesion molecules is involved in the formation of blood-borne metastasis. Such a process may initiate migration of tumor cells through the endothelium into underlying tissues and thus tumor cells cannot be destroyed by the immune system. Although several adhesion molecules may be involved in this process, it appears that the overexpression of ICAM-1 (intracellular adhesion molecule-1) may play a critical role in breast cancer metastatic formation.

In our research we are the first to propose that lipid-enhanced breast cancer metastasis may be connected to the overexpression of ICAM-1. The fact that a variety of fatty acids have different effects on ICAM-1 induction may explain different effects of dietary lipids on breast cancer metastasis. In the current grant application, we propose to study mechanisms of lipid-induced ICAM expression and breast tumor cell metastatic formation on molecular, cellular and whole animal levels.

Our research in year 1 was solely based on endothelial cell culture model system and treatments including different dietary fatty acids.

2. BODY

a. Research accomplishments associated with Task 1.

Task 1. **To identify the specific phosphorylation mechanism involved in lipid-mediated induction of ICAM-1 expression.**

The results obtained as a result of this Task indicate that exposure to linoleic acid increases protein kinase C (PKC) and mitogen-activated protein (MAP) kinase activities. In addition, inhibition of both PKC and MAP-kinase prevented linoleic acid-mediated activation of NF-κB. Endothelial cell exposure to linoleic acid also decreased cAMP levels, which indicates that c-AMP-dependent protein kinase (PKA) is an unlikely participant in fatty acid-mediated activation of NF-κB. Thus, in this research we identified two specific signal transduction mechanisms responsible for fatty acid-mediated activation of NF-κB. Which of these two pathways plays more important role in fatty acid-mediated activation of NF-κB and ICAM-1 gene expression requires further studies. Such studies may involve transfections of endothelial cells with specific NF-κB as well as IκB reporter contracts. Because endothelial cells are well known to be difficult to transfect, we developed a special technique which allows us to achieve a high-efficiency transfection of human endothelial cells. This technique was recently published by our group (Kaiser and Toborek J. Vasc. Res. 38:133-143, 2001) and it constitutes another major accomplishment resulting from this grant proposal. We were the first to report that transfection of endothelial cells can achieve as high as 32% efficiency (Figure 1). This technique also was employed in our research on NF-κB-mediated induction of ICAM-1 gene, as well as other inflammatory genes in human endothelial cells (Toborek et al., Am. J. Clin. Nutr., in press., Park at al., submitted).

Detailed descriptions of the obtained results are included in the appended publications:

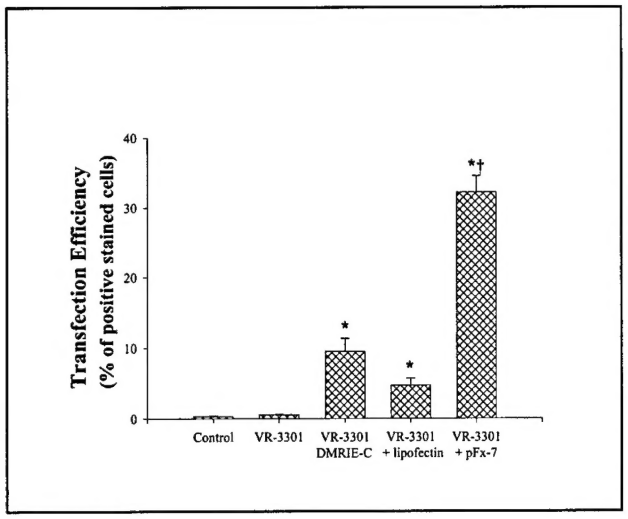

Figure 1. Efficiency of liposome-mediated transfection of human endothelial cells. Cells were transfected for 1.5 h with the VR-3301 vector (5 μg/mL) complexed with 40 μg/mL of DMRIE-C or lipofectin or with 36 μg/mL of pFx-7. *Values marked with an asterisk are significantly higher as compared to the values for control cultures or cultures transfected with naked plasmid DNA. †Values in cultures transfected in the presence of pFx-7 are significantly higher than values in other experimental groups.

b. Research accomplishments associated with Task 2.

Task 2. **To test the hypothesis that induction of ICAM-1 expression mediated by polyunsaturated but not saturated fatty acids, is the critical factor in promoting adhesion of breast tumor cells to endothelial cells and their transendothelial migration.**

Extensive studies were performed in relationship to this Task. We indicated that dietary fatty acids can exert specific effects on ICAM-1 gene expression. Exposure to both linoleic acid and linolenic acid induced a dose dependent increase in ICAM-1 mRNA levels. In addition, these two fatty acids at the concentration of 90 μmol/L stimulated induction of the ICAM-1 gene to a similar extent, i.e., by approximately 30% as measured by the density of the appropriate fluorescent bands. In contrast, exposure of endothelial cells to oleic acid decreased ICAM-1 mRNA levels to approximately 50% of control values. The results of these experiments are shown in Figure 2. The full report on dietary fatty acid-mediated expression of inflammatory genes in human endothelial cells was recently accepted for publication in the American Journal of Clinical Nutrition (Toborek et al., Am. J. Clin. Nutr., in press.) and is also appended to this Progress Report.

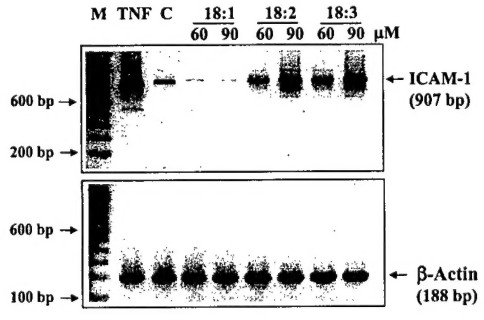

Figure 2C. Effects of dietary fatty acids on intercellular adhesion molecule-1 (ICAM-1) mRNA levels in human endothelial cells as measured by RT-PCR. Endothelial cells were exposed to specific fatty acids for 3 hours. β-Actin was determie to indicate that the same amount of RNA was used per sample.

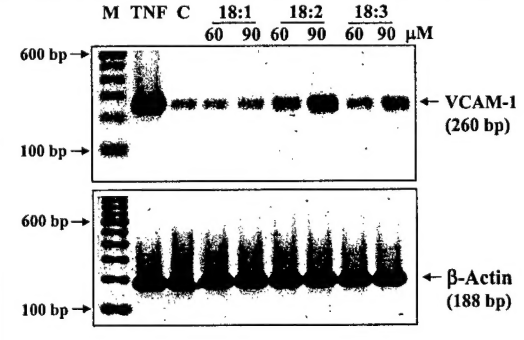

Figure 2C. Effects of dietary fatty acids on vascular cell adhesion molecule-1 (VCAM-1) mRNA levels in human endothelial cells as measured by RT-PCR. Endothelial cells were exposed to specific fatty acids for 3 hours. β-Actin was determie to indicate that the same amount of RNA was used per sample.

It appears that not only ICAM-1 but also another adhesion molecule, namely vascular cell adhesion molecule-1 (VCAM-1), may play an important role in dietary fatty acid-mediated cancer metastasis. The effects of specific unsaturated fatty acids on VCAM-1 mRNA levels in HUVEC are indicated in Figure 3. The most significant induction of the VCAM-1 gene (by 38% as measured by the density of the fluorescent bands) was observed in cells treated with 90 μmol/L of linoleic acid. In addition, exposure to 90 μmol/L of linolenic acid resulted in a slight increase in VCAM-1 mRNA levels. Treatment with oleic acid had no effect on VCAM-1 gene induction as compared to control cultures.

Because of profound effects of linoleic acid on VCAM-1 gene expression, detailed studies were performed on the mechanisms of this process. We indicated that the NF-κB binding site plays the critical role in linoleic acid-induced VCAM-1 gene expression in human endothelial cells. In addition, we indicated that common anti-inflammatory drugs, such as aspirin or sodium salicylate can inhibit linoleic acid-mediated activation of NF-κB (Figure 4) as well as linoleic acid-induced VCAM-1 expression (Figure 5).

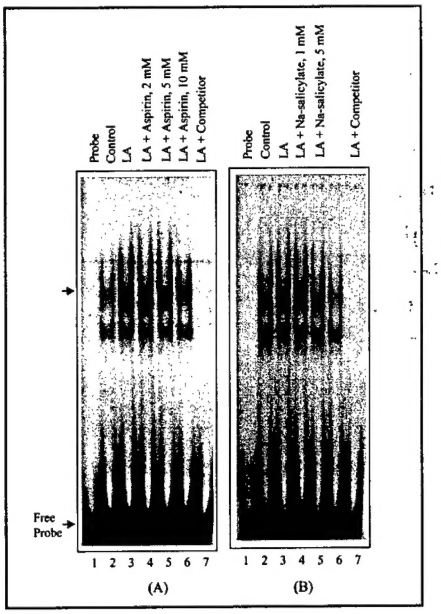

Figure 4. Pretreatment with aspirin, sodium salicylate or PDTC blocks linoleic acid (LA)-induced NF-κB DNA-binding activity in human microvascular endothelial cells (HMEC-1) as measured by EMSA. HMEC-1 were pretreated for 1 h with indicated concentrations of (A) aspirin or (B) sodium salicylate before a 2 h treatment with 50 μM of linoleic acid (lanes 4-6). Lane 1, probe alone; lane 2, treatment with 50 μM linoleic acid alone; lane 7, competition study performed by the addition of excess unlabeled oligonucleotide using nuclear extract from cells treated with 50 μM linoleic acid.

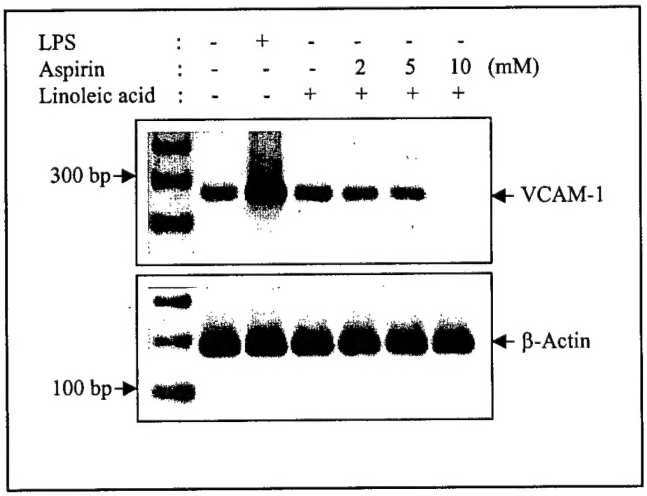

Figure 5. Pretreatment with aspirin impedes the induction of VCAM-1 mRNA expression in linoleic acid-treated human microvascular endothelial cells (HMEC-1). Cells were pretreated for 1 h with indicated concentrations of aspirin for 30 min with PDTC, before a 4 h treatment with 50 μM of linoleic acid and assayed for VCAM-1 mRNA expression by RT-PCR. LPS (1 μg/mL) was used as positive control.

Detailed descriptions of the obtained results are included in the appended publications:

b. Research accomplishments associated with Task 3.

Task 3. **To test the hypothesis that diets enriched with polyunsaturated dietary fats but not saturated fats increase metastasis formation and breast tumor development in an animal model by induction of ICAM-1 expression.**

Animal studies will be performed in year 3 of this grant proposal.

3. KEY RESEARCH ACCOMPLISHMENTS

- To identify two specific phosphorylation pathways which are induced by dietary fatty acids and participate in fatty acid-mediated activation of NF-κB.
- To establish a new transfection technique which allows the transfection of human endothelial cells with a high efficiency.
- To indicate that antioxidants and common anti-inflammatory drugs, such as aspirin, can inhibit dietary fatty acid-mediated activation of NF-κB and adhesion molecule expression in endothelial cells.
- To determine that the NF-κB binding site plays the critical role in linoleic acid-induced expression of adhesion molecules in human endothelial cells

4. REPORTABLE OUTCOMES

a. REFEREED ARTICLES

Park HJ, Lee YW, Hennig B, Toborek M: Linoleic acid-induced VCAM-1 expression in human microvascular endothelial cells is mediated by the NF-κB-dependent pathway. (submitted)

Toborek M, Lee YW, Kaiser S, Hennig B: Inflammatory properties of fatty acids. Methods in Enzymology (CK Sen and L Packer; eds.) in press.

Toborek M, Lee YW, Garrido R, Kaiser S, Hennig B: Unsaturated fatty acids selectively induce an inflammatory environment in human endothelial cells. Am. J. Clin. Nutr., in press.

Kaiser S, Toborek M: Liposome-mediated high-efficiency transfection of human endothelial cells. J. Vasc. Res. 38, 133-143, 2001.

Hennig B, Toborek M: Nutrition and endothelial cell function: implications in atherosclerosis. Nutr. Res. 21, 279-293, 2001.

Hennig B, Toborek M, McClain CJ: High-energy nutrients, fatty acids and endothelial cell function: implications in atherosclerosis. J. Am Coll. Nutr. 20, 97-105, 2001.

Hennig B, Meerarani P, Ramadass P, Watkins BA, Toborek M: Fatty acid-mediated activation of vascular endothelial cells. Metabolism 49, 1006-1013, 2000.

Hennig B, Toborek M, Boissonneault GA: Lipids, inflammatory cytokines, and endothelial cell Injury. In: Nutrition and Immunology: Principle and Practice. (ME Gershwin, B German, C Keen, editors), Humana Press, Inc., Totowa, NJ, 203-220, 2000.

b. PRESENTATIONS/ABSTRACTS

Park H-J, Lee YW, Hennig B, Toborek M: Linoleic acid-induced VCAM-1 expression in human microvascular endothelial cells is mediated by the NF-kB-dependent pathway. FASEB J. 15, A866, 2001.

Saraswathi V, Narayan P, Hammock BD, Meerarani P, Toborek M, Hennig B. Linoleic acid-derived epoxides alter calcium and nitric oxide metabolism in endothelial cells. FASEB J. 15, A190, 2001.

Slim RM, Hammock BD, Toborek M, Robertson LW, Watkins BA, Hennig B: The role of methyl linoleic acid epoxide and diol metabolites in the synergistic toxicity of linoleic acid and PCBs to vascular endothelial cells. Toxicol. Sci., 60, 13, 2001.

Hennig B, Toborek M: High-energy nutrients, fatty acids and endothelial cell function: implications in atherosclerosis. J. Am. Coll. Nutr. 19, 678, 2000.

Toborek M, Lee YW, Garrido R, Kaiser S, Hennig B. Dietary fatty acid-induced oxidative and inflammatory environments in endothelial cells. J. Am. Coll. Nutr. 19, 684, 2000.

Hennig B, Meerarani P, Kaiser S, Toborek M: Inflammatory events and fatty acid-induced activation of vascular endothelial cells. FASEB J. 14, A199, 2000.

Meerarani P, Ramadass P, Toborek M, Keller J, Hennig B: The role of different fatty acids in oxidative injury and dysfunction of endothelial cells. FASEB J. 13, A1117, 1999.

Ramadass P, Meerarani P, Toborek M, Bauer HC, Bauer H, Probst G, McClain CJ, Hennig B: Protective effects of zinc against endothelial cell apoptosis induced by linoleic acid and/or TNF. FASEB J. 13, A832, 1999.

Kaiser S, Toborek M: High-efficiency transfection of human endothelial cells mediated by cationic lipids. Endothelium, in press.

Hennig B, Toborek M: Fatty acid-induced endothelial cell activation: Implications in atherosclerosis. Pol. J. Pharmacol., in press.

5. CONCLUSIONS

Our studies have demonstrated that dietary fatty acids can exert highly specific effects on NF-κB activation and expression of adhesion molecules in human endothelial cells. In addition, we indicated that linoleic acid induces ICAM-1 and VCAM-1 expression through the activation

of NF-κB. More importantly, because the ability of cancer cells to interact with the endothelium appears to be a prerequisite for the potential of distant metastasis and because ICAM-1 and VCAM-1 are considered to be a crucial adhesion molecule in this process, the present study may have significant therapeutic implications in developing a novel strategy against cancer metastasis. These studies also provide a mechanistic insight of the role of specific dietary lipids in metastasis. Therefore, data arising from this grant proposal may allow dietary and molecular intervention to protect against breast cancer metastasis.

6. Proposed changes in time efforts

I would like to reduce my time effort on this research grant. Originally, I estimated that 20% of my time effort would be needed to complete this proposal. However, at the present time, I would like to reduce my time effort to 5%.

My initial time effort included the training of research personnel in my laboratory. However, I am now able to delegate some of my initial research activities to my postdoctoral scholars, in particular to Dr. Yong Woo Lee, and to laboratory technicians. Therefore, despite reduction of my time effort, all experiments designed in our proposal will be fully completed and I will continue to be very actively involved in this research.

Dr. Yong Woo Lee is a Research Associate currently assigned to this proposal. He has been working in my laboratory already for 3 years. During that time, Dr. Lee received training in molecular biology and he is now fully familiar with all aspects of the proposed research. In fact, he is the first author on several manuscripts which were submitted or published. All these manuscript are based on endothelial cell culture systems, i.e., the same cell culture models which are designed in our active award. Therefore, I believe that Dr. Lee's current expertise allows me to reduce my time effort on this grant proposal without compromising the designed experiments. Finally, both Dr. Lee and myself were recently promoted. Thus, any change in salary due to the reduction of effort will be absorbed as a result of our promotions.

Recent publications and abstracts from my laboratory on which Dr. Lee is the first author.

REFEREED ARTICLES

Lee YW, Kühn H, Kaiser S, Hennig B, Daugherty A, Toborek M: Interleukin-4 induces transcription of the 15-lipoxygenase I gene in human endothelial cells. J. Lipid Res. 42, 783-791, 2001.

Lee YW, Kühn H, Hennig B, Neish AS, Toborek M: IL-4-induced oxidative stress upregulates VCAM-1 gene expression in human endothelial cells. J. Mol. Cell. Cardiol. 33, 83-94, 2001.

Lee YW, Kühn H, Hennig B, Toborek M: Interleukin-4 induces apoptosis of human endothelial cells through the caspase-3-dependent pathway. FEBS Lett. 485, 122-126, 2000.

Lee YW, Hennig B, Toborek M: IL-4 induces STAT1a-mediated monocyte chemoattractant protein-1 expression by antioxidant-sensitive mechanism in human vascular endothelial cells. (submitted)

Lee YW, Hennig B, Yao J, Toborek M: Methamphetamine induces AP-1 and NF-kB binding and transactivation in human brain endothelial cells. (submitted)

Lee YW, Hennig B, Fiala M, Toborek M: Cocaine activates redox-regulated transcription factors and induces TNF-a expression in human brain endothelial cells. (submitted)

ABSTRACTS

Lee YW, Nath A, Hennig B, Toborek M: Inflammatory environments induced by methamphetamine in human brain endothelial cells. Neurology 56, Suppl 3, A166, 2001.

Lee YW, Kühn H, Hennig B, Toborek M: Interleukin-4 induces apoptosis of human endothelial cells through the caspase-3-dependent pathway. FASEB J. 15, A510, 2001.

Lee YW, Kühn H, Hennig B, Toborek M: IL-4-induced VCAM-1 gene expression human endothelial cells by oxidative mechanisms. FASEB J. 14, A1545, 2000.

Lee YW, Kühn H, Hennig B, Daugherty A, Toborek M: Intereukin-4-mediated transcriptional regulation of 15-lipoxygenase gene expression in HUVEC. Mol. Biol. Cell 10 (Suppl), 324a, 1999.

LINOLEIC ACID-INDUCED VCAM-1 EXPRESSION IN HUMAN MICROVASCULAR ENDOTHELIAL CELLS IS MEDIATED BY THE NF-κB-DEPENDENT PATHWAY

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ABSTRACT

Vascular cell adhesion molecule-1 (VCAM-1) has been reported to play an important role in cancer metastasis *via* the adhesive interaction between tumor cells and endothelial cells. In this study, we examined the effects of linoleic acid on VCAM-1 expression and its transcriptional regulatory mechanism in human microvascular endothelial cells (HMEC-1). Time- and dose-dependent increases of VCAM-1 mRNA levels were observed in linoleic acid-treated HMEC–1 as detected by RT-PCR. Flow cytometry analysis showed a significant and dose-dependent upregulation of VCAM-1 expression in HMEC-1 stimulated with linoleic acid as compared to controls. In order to clarify the transcriptional regulatory pathway, we investigated the role of nuclear factor-κB (NF-κB) in the expression of VCAM-1 by linoleic acid in HMEC-1. Nuclear extracts from HMEC-1 stimulated with linoleic acid showed a dose-dependent increase in binding activity to the NF-κB consensus sequences. These effects were preventable by co-treatment with inhibitors of NF-κB activity, such as sodium salicylate, aspirin or pyrrolidine dithiocarbamate (PDTC). In addition, pretreatment with NF-κB inhibitors markedly suppressed the ability of linoleic acid to induce VCAM-1 gene expression. The role of NF-κB in linoleic acid-induced VCAM-1 expression was confirmed by functional promoter studies in HMEC-1 transfected with reporter constructs of the VCAM-1 promoter with or without mutated NF-κB binding site. These results indicate that linoleic acid upregulates VCAM-1 expression in HMEC-1 through the NF-κB-dependent pathway.

Key words: metastasis, adhesion molecules, dietary fatty acids, transcriptional regulation, vascular endothelium.

Abbreviations: Electrophoretic mobility shift assay, EMSA; human microvascular endothelial cells, HMEC-1; interleukin, IL; lipopolysaccharide, LPS; nuclear factor-κB, NF-κB; polyunsaturated fatty acids, PUFAs; pyrrolidine dithiocarbamate, PDTC; tumor necrosis factor-α, TNF-α; vascular cell adhesion molecule-1, VCAM-1; very late antigen-4, VLA-4.

INTRODUCTION

Dietary fat is considered to be one of the main risk factors of carcinogenesis. For example, a positive correlation was reported between dietary fat intake and increased risks for the development of breast, colon and prostate cancers (1-3). Although the role of individual fatty acids in cancer development is not fully understood, evidence has shown that vegetable oils rich in ω-6 polyunsaturated fatty acids (PUFAs), such as linoleic acid (C18:2, ω-6) can promote mammary carcinogenesis whereas similar levels of marine oils rich in ω-3 PUFAs appear to inhibit this process (4). To further support the role of linoleic acid in carcinogenesis, it was demonstrated that when dietary content of this fatty acid exceeded 4-5% of total calories, any additional fat linearly increased chemically-induced tumor incidence (5,6). Moreover, mammary tumors developed more readily when a diet based on saturated fats, such as coconut oil or beef tallow, was enriched with linoleic acid (7). In addition to its role in carcinogenesis, dietary linoleic acid can also enhance the metastatic formation of mammary tumors. For example, a linoleic acid-enriched diet increased the rate of metastasis of mammary cancer to the lung in rats (8).

The formation of blood-borne metastasis is a complex process which requires several steps. However, a growing body of evidence indicates that the direct adhesive interaction between tumor cells and endothelial cells is the critical event in metastasis formation (9,10). This process requires the binding of tumor cells to specific adhesion molecules on the surface of endothelial cells, followed by migration of tumor cells through the endothelium into underlying tissues (9). Evidence indicates that among several adhesion molecules which can be involved in this process, vascular cell adhesion molecule-1 (VCAM-1) may play one of the most important roles. For example, it was demonstrated that VCAM-1 facilitated adhesion of metastatic breast tumor cells to endothelial cells stimulated by shear stress (11). In patients with breast cancer or gastric cancers, serum levels of soluble VCAM-1 were closely correlated with disease progression (12,13). The role of VCAM-1 in breast cancer metastasis was supported by the observation that the expression of this adhesion molecule was increased on breast tumor endothelium (14). Upregulation of VCAM-1 was also shown to be involved in adhesion of RAW117 lymphoma cells (15) or melanoma cells to hepatic sinusoidal endothelial cells (16). In addition, evidence indicated the role of VCAM-1 in adhesion of B9/BM1 cells to bone marrow-

derived endothelial cells (17), and adhesion of DU145 cells (the cell line derived from cerebral metastasis of prostate carcinoma) to human brain microvascular endothelial cells (18).

VCAM-1 is a 110 kDa member of the immunoglobulin gene superfamily first described as a cytokine-inducible endothelial adhesion protein (19). It facilitates tumor cell adhesion through binding of an integrin counter receptor, very late antigen-4 (VLA-4) (20). Functional studies on the activity of the VCAM-1 gene promoter have shown that regulation of VCAM-1 gene expression in endothelial cells appears to be complex and related to the type of stimuli. For example, VCAM-1 induction by inflammatory cytokines, such as interleukin (IL)-1β or tumor necrosis factor-α (TNF-α), as well as by lipopolysaccharide (LPS) strongly relies on activation of nuclear transcription factor-κB (NF-κB) (21,22). In contrast, recent evidence indicated that IL-4-induced VCAM-1 expression is independent of NF-κB activation (23,24). Thus, the specific role of NF-κB in linoleic acid-induced overexpression of the VCAM-1 gene is uncertain and was chosen as the subject of the present study.

Because of the significance of dietary linoleic acid and VCAM-1 expression in cancer metastasis, the aim of the present study was to investigate the molecular signaling pathways involved in linoleic acid-induced VCAM-1 upregulation in human microvascular endothelial cells. We have determined that linoleic acid can induce VCAM-1 expression at the mRNA and protein levels. Furthermore, we provide evidence that linoleic acid-stimulated expression of the VCAM-1 gene is mediated by activation of NF-κB.

MATERIALS AND METHODS

Cell culture

Human microvascular endothelial cells (HMEC-1) were a generous gift from Dr. Eric Smart (University of Kentucky Medical Center). HMEC-1 were cultured in MCDB 131 media (Sigma, St. Louis, MO) enriched with 10% fetal bovine serum, 1% penicillin/streptomycin, 1 μg/ml hydrocortisone and 0.01 μg/ml epidermal growth factor in a 5% CO_2 atmosphere at 37 °C. Linoleic acid (>99% pure) was obtained from Nu-Chek Prep (Elysian, MN). The medium was enriched with linoleic acid as described previously (25).

In selected experiments, HMEC-1 were pretreated for 1 h with salicylates (aspirin or sodium salicylate), or for 30 min with pyrrolidine dithiocarbamate (PDTC). Salicylates were used at concentrations of up to 10 mM and PDTC was employed at levels of up to 25 μM.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts from HMEC-1 were prepared according to the method of Beg *et al* (26). Binding reactions were performed in a 20 μl volume containing 4 ~ 10 μg of nuclear protein extracts, 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.1 mM dithiothreitol, 10% glycerol, 2 μg of poly[dI-dC] (nonspecific competitor) and 40,000 cpm of ^{32}P-labeled specific oligonucleotides that contained the consensus sequence for NF-κB site (5'-AGTTGAGGGGACTTTCCCAGG-3'). The resultant protein-DNA complexes were resolved on native 5% polyacrylamide gels using 0.25 × TBE buffer (50 mM Tris-Cl, 45 mM boric acid, 0.5 mM EDTA, pH 8.4). Competition studies were performed by the addition of a molar excess of unlabeled oligonucleotide to the binding reaction. Rabbit polyclonal anti-p50 and anti-p65 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were employed in supershift experiments.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted by the use of TRI reagent (Sigma, St. Louis, MO) and reverse-transcribed at 42 °C for 60 min in 20 μl of 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1 mM dNTP, 1 unit/μl of recombinant RNasin ribonuclease inhibitor, 15 units/μg of AMV reverse transcriptase, and 0.5 μg of oligo$(dT)_{15}$ primer. For amplification of VCAM-1 and of β-actin (a housekeeping gene), the following primer combinations were used: 5'-ATGACATGCTTGAGCCAGG-3' and 5'-GTGTCTCCTTCTTTGACACT-3' (VCAM-1; expecting 260-bp fragment) (27) and 5'-AGCACAATGAAGATCAAGAT-3' and 5'-TGTAACGCAACTAAGTCATA-3' (β-actin; expecting 188-bp fragment) (28). The PCR mixture consisted of a Taq PCR Master Mix Kit (Qiagen, Valencia, CA), 2 μl of the reverse transcriptase reaction, and 20 pmol of primer pairs in a total volume of 50 μl. Thermocycling was performed according to the following profile: 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, repeated 20 times. Amplification was linear within the range of 15-30 cycles. PCR

products were separated by 2% agarose gel electrophoresis, stained with SYBR® Green I (Molecular Probes, Eugene, OR) and visualized using phosphoimaging technology (FLA-2000, Fuji, Stamford, CN).

Flow cytometry for VCAM-1 determination

VCAM-1 protein expression was determined by flow cytometry. Briefly, HMEC-1 were grown to confluence on six-well culture plates and treated with linoleic acid for 12 h. HMEC-1 were then washed with Hank's buffer and gently harvested by trypsin/EDTA. Cells were washed twice with PBS and incubated for 1 h on ice with saturating amounts of monoclonal mouse anti-human VCAM-1 antibody labeled with FITC (R&D Systems, Minneapolis, MN). FITC-labeled mouse anti-human IgG1 was used as the isotype control (R&D Systems). After incubation with antibodies, samples were washed twice with ice-cold PBS and analyzed with 10,000 cells per sample in a fluorescence-activated cell sorter (Becton Dickinson, San Jose, CA). Following correction for unspecific binding (isotype control), specific mean fluorescence intensity was expressed as the indicator of VCAM-1 protein expression.

Transient transfection and reporter gene assays

Transient transfections of HMEC-1 were performed using pFx-7 (Invitrogen, Carlsbad, CA) as described earlier (29). Cells were transfected with 10 μg of the VCAM-1 promoter constructs with or without mutated NF-κB site (pF3-mNF-κB-CAT3 and pF3-CAT3, respectively) and co-transfected with 4 μg of the pGL3-Luc control vector (Promega, Madison, WI) to normalize transfection rates. The reporter constructs, pF3-mNF-κB-CAT3 and pF3-CAT3, were kind gifts from Dr. Andrew S. Neish (Emory University School of Medicine). Generation of these constructs was described and characterized earlier (21,30). Following transfection, cultures were maintained in normal growth medium for 24 h and then exposed to 50 μM of linoleic acid for an additional 24 h in MCDB131 medium enriched with 5% FBS. After treatment exposure, cells were washed twice with phosphate buffered saline and lysed in 100 μl of Reporter Lysis Buffer (Promega). Chloramphenicol acetyltransferase (CAT) activity was determined using the method of Gorman *et al* (31). The cell lysates, normalized for protein levels, were incubated for 4 h at 37 °C with a reaction mixture composed of 125 mM Tris-HCl (pH 7.8), 0.83 mM acetyl coenzyme A, and 3 μl of [^{14}C]chloramphenicol (25 μCi/ml; Amersham

Pharmacia Biotech, Piscataway, NJ). Then, acetylated and nonacetylated forms of chloramphenicol were extracted with ethyl acetate and separated by thin-layer chromatography using the solvent system with chloroform:methanol (95:5, v/v). Following autoradiography, the zones corresponding to acetylated or non-acetylated chloramphenicol were cut from the plates and radioactivity was counted in a liquid scintillation counter for quantitation of CAT activity. The CAT activity was normalized according to luciferase activity, which was determined using Luciferase Assay System (Promega) according to the manufacturer's protocol.

Statistical analysis

Routine statistical analysis of data was completed using SYSTAT 7.0 (SPSS Inc., Chicago, IL). One-way ANOVA was used to compare mean responses among the treatments. The treatment means were compared using Bonferroni least significant difference procedure. Statistical probability of $p<0.05$ was considered significant.

RESULTS

Linoleic acid activates NF-κB in HMEC-1

To determine if linoleic acid can activate NF-κB in HMEC-1, cells were exposed to this fatty acid for 2 h and NF-κB binding was analyzed by EMSA, using nuclear extracts of the treated cells. Results of these experiments are shown in Figures 1A and 1B. Figure 1A depicts the effects of linoleic acid on the binding activity of NF-κB in HMEC-1. A slight endogenous activity of NF-κB was observed in control cultures (Figure 1A, lane 2). However, when the cells were stimulated with linoleic acid, a marked increase in NF-κB binding activity was detected (lanes 4-6). This binding was specifically inhibited by an unlabeled competitor DNA containing the consensus NF-κB sequence (lane 7). In addition, the identity of NF-κB binding was confirmed by supershift experiments with antibodies against specific NF-κB subunits, p50 and p65 (Figure 1B, lanes 3 and 4).

To further study linoleic acid-induced activation of NF-κB, HMEC-1 were pretreated for 1 h with aspirin or sodium salicylate or for 30 min with PDTC before a co-exposure to linoleic acid for 2 h. Both salicylates and PDTC are widely used as inhibitors of NF-κB activation.

Indeed, as indicated in Figure 2, pretreatment with aspirin (Figure 2A), sodium salicylate (Figure 2B) or PDTC (Figure 2C) resulted in dose-dependent inhibitions of NF-κB activation in HMEC-1 exposed to linoleic acid.

Linoleic acid induces VCAM-1 expression in HMEC-1

To investigate whether exposure to linoleic acid can induce expression of VCAM-1 in microvascular endothelial cells, HMEC-1 were treated with 50 μM linoleic acid for increasing time periods and the VCAM-1 mRNA level was determined by semi-quantitative RT-PCR technique. As shown in Figure 3, low levels of constitutively expressed VCAM-1 mRNA were detected in control cells (no linoleic acid supplementation). On the other hand, mRNA transcripts for VCAM-1 were clearly increased in linoleic acid-treated cells. Upregulation of VCAM-1 expression was already observed after a 1 h exposure to linoleic acid, reaching maximal levels at 4 h (Figure 3A).

Figure 3B shows that a 4 h exposure of HMEC-1 to linoleic acid resulted in a dose-dependent increase in the VCAM-1 mRNA. The most marked VCAM-1 expression was observed in HMEC-1 cultures treated with linoleic acid at the dose of 50 μM. Additional increase in linoleic acid concentration did not further potentiate VCAM-1 expression (data not shown).

Figure 4 indicates the effects of increasing concentrations of linoleic acid treatment on VCAM-1 protein expression as measured by flow cytometry. In agreement with RT-PCR data, VCAM-1 protein was constitutively expressed in untreated HMEC-1. However, in cells treated with linoleic acid for 12 h, expression of this adhesion molecule was markedly upregulated in a dose-dependent manner.

Linoleic acid-induced VCAM-1 expression in HMEC-1 is mediated by NF-κB

To determine if linoleic acid-mediated activation of NF-κB is involved in upregulation of VCAM-1, expression of the VCAM-1 gene was studied in HMEC-1 pretreated with different doses of NF-κB inhibitors and exposed to 50 μM linoleic acid for 4 h. Similar to our studies presented in Figure 2, aspirin, sodium salicylate, and PDTC were employed to inhibit NF-κB. Effects of these NF-κB inhibitors on linoleic acid-induced overexpression of the VCAM-1 gene are reflected in Figure 5. As indicated, a 1 h pretreatment with increasing doses of aspirin

(Figure 5A) or sodium salicylate (Figure 5B), as well as a 30 min pretreatment with PDTC (Figure 5C), markedly and in a dose-dependent manner decreased linoleic acid-mediated stimulation of the VCAM-1 gene.

To further determine that the NF-κB binding site plays the critical role in linoleic acid-induced VCAM-1 gene expression in HMEC-1, cells were transfected with the construct of normal VCAM-1 promoter (pF3-CAT3) or with a similar construct that had a mutated NF-κB binding site (pF3-mNF-κB-CAT3). As indicated in Figure 6, exposure to linoleic acid induced CAT activity only in cells transfected with the pF3-CAT3 construct. In contrast, mutation of the NF-κB site completely inhibited linoleic acid-induced stimulation of CAT activity in HMEC-1 transfected with the pF3-mNF-κB-CAT3 construct.

DISCUSSION

Adhesive interactions between vascular endothelial cells and tumor cells play a critical role in the process of metastatic tumor dissemination. This process is mediated by adhesion molecules, which are expressed on the surface of endothelial cells, and specific integrin receptors, which are present on tumor cells. Following adhesion, tumor cells can migrate across the vascular endothelium and establish new metastatic colonies. It appears that among different adhesion molecules involved in endothelial cell-tumor cell interactions, VCAM-1 may play one of the most important roles (12-18). In addition, clinical and animal studies have implicated the intake and composition of dietary fats in expression of endothelial cell adhesion molecules, including upregulation of VCAM-1 (32-34).

In the average American diet, fat accounts for 35-40% of energy (35), and such overconsumption of foods rich in fat may be a major risk both for cancer development and metastasis. In fact, dietary factors, including excessive intake of fat, can contribute to 35% of all cancers (1). Part of the carcinogenic and prometastatic effects of dietary fat can be related to modulation of the functions of the vascular endothelium. It appears that among different dietary fatty acids, linoleic acid can alter endothelial cell metabolism most significantly (36) and thus induce the development of cancer metastasis (5,6,8). Since it is an unsaturated fatty acid, linoleic acid can undergo peroxidative pathways initiated by hydrogen abstraction followed by

oxygen attack on the generated lipid alkyl radical (37). In fact, several reports suggest that linoleic acid can act as a potent prooxidant in endothelial cells in culture. For example, linoleic acid can enhance radical adduct formation in endothelial cells exposed to iron-induced oxidative stress (38), decrease glutathione levels (25), and increase peroxisomal β-peroxidation (39), a pathway that leads to the production of hydrogen peroxide. Degradation of linoleic acid *via* peroxidative pathways also can lead to formation of highly cytotoxic products, such as linoleic acid hydroperoxides or 4-hydroxy-2-(E)-nonenal (40). However, to date, vascular effects of linoleic acid have been only studied in cells isolated from major vascular vessels, such as pulmonary artery (41,42) or umbilical veins (43). It is well known that the structure and functions of endothelial cells that originated from different tissues and vessels can differ markedly (44). Therefore, the present study focused on mechanistic effects of linoleic acid on induction of VCAM-1 in human microvascular endothelial cells, i.e., in the cell type which is most relevant to cancer metastasis.

In the present study we report that treatment of HMEC-1 with linoleic acid results in an increase of the steady state concentration of the VCAM-1 mRNA in a time- and dose-dependent manner (Figure 3). In addition, flow cytometry analysis showed that linoleic acid-induced upregulation of the VCAM-1 gene is correlated with a significant and dose-dependent overexpression of VCAM-1 protein in HMEC-1 (Figure 4). These results are in agreement with earlier reports which indicated upregulation of another adhesion molecule, such as intercellular adhesion molecule-1 (ICAM-1), in endothelial cells treated with linoleic acid (43). Recent evidence also indicated that an oxidized derivative of linoleic acid, 13-hydroperoxy-octadecadienoic acid (13-HPODE), can induce VCAM-1 gene expression in endothelial cells (45). On the other hand, a 72 h pre-exposure of endothelial cells to selected n-3 or n-6 fatty acids, followed by a co-treatment with IL-1β or TNF-α for an additional 12 h, resulted in an inhibition of cytokine-induced VCAM-1 expression as compared to cells which were not pretreated with fatty acids (46). However, a very different experimental setting used in that study was, most likely, responsible for this discrepancy with our present results.

The current study also reveals that treatment of HMEC-1 with linoleic acid can activate NF-κB in a dose-dependent manner. These results are in agreement with earlier reports on NF-κB activation by linoleic acid in porcine pulmonary artery endothelial cells (41,42). It is possible that linoleic acid-mediated induction of oxidative stress (42), a decrease in cellular

glutathione (25) and alterations of cellular redox status (25,42) are responsible for activation of NF-κB. To support the role of oxidative stress in linoleic acid-induced activation of NF-κB, this effect was attenuated by salicylates and PDTC (Figure 2). Aspirin and sodium salicylate have been previously shown to specifically inhibit the activation of NF-κB by preventing the degradation of IκB, a NF-κB inhibitory subunit, and blocking the translocation of NF-κB into the nuclear compartment (47,48). PDTC, the radical-scavenging thiol compound, is also widely used as an inhibitor of NF-κB activation (49,50).

NF-κB binding sites are located in the promoter regions of the genes encoding for adhesion molecules, including VCAM-1 (51). In fact, two adjacent κB sites located at the positions -77 and -63 relative to the transcription start site were identified in the VCAM-1 promoter (21,22). The role of these κB binding sites in the induction of the VCAM-1 gene is not fully understood and may depend on the type of stimulus. For example, NF-κB binding appears to be critical in TNF-α or LPS-induced VCAM-1 expression (21,22,51). In contrast, IL-4-mediated induction of the VCAM-1 gene is independent of NF-κB activation. This phenomenon was reported both in endothelial cells (23) and in other types of vascular cells (24). These conflicting reports on the role of NF-κB activation in VCAM-1 gene expression prompted us to investigate the role of this transcription factor in linoleic acid-mediated stimulation of VCAM-1 in HMEC-1. In the present study, two different lines of experiments proved that linoleic acid-induced activation of NF-κB and induction of the VCAM-1 gene are interrelated. First, pretreatment of the HMEC-1 with inhibitors of NF-κB activation, such as salicylates or PDTC, completely inhibited linoleic acid-induced VCAM-1 expression (Figure 5). Second, reporter gene assays were performed using normal VCAM-1 promoter reporter construct as well as similar construct but with mutated NF-κB binding site. As indicated in Figure 6, mutation of the NF-κB binding site in the VCAM-1 promoter region completely abolished linoleic acid-induced expression of the reporter gene. These results specifically indicate the importance of NF-κB activation in linoleic acid-induced expression of the VCAM-1 gene.

In conclusion, our studies have demonstrated that linoleic acid induces VCAM-1 expression in human microvascular endothelial cells through the activation of transcription factor NF-κB. More importantly, because the ability of cancer cells to interact with the endothelium appears to be a prerequisite for the potential of distant metastasis and because VCAM-1 is

considered to be a crucial adhesion molecule in this process, the present study may have significant therapeutic implications in developing a novel strategy against cancer metastasis. Finally, these studies provide a mechanistic insight of the role of specific dietary lipids in metastasis.

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FIGURE LEGENDS

Figure 1A. Linoleic acid (LA) treatment enhances NF-κB binding in human microvascular endothelial cells (HMEC-1) as analyzed by EMSA. HMEC-1 were either untreated (lane 2) or treated for 2 h with increasing doses of linoleic acid (lanes 4-6). Competition study was performed by the addition of excess unlabeled oligonucleotide (lane 7) using nuclear extracts from cells treated with 50 μM linoleic acid. Lane 1, probe alone; lane 3, LPS (1 μg/mL, positive control).

Figure 1B. Supershift analysis of linoleic acid (LA)-induced NF-κB binding activity in human microvascular endothelial cells (HMEC-1). Nuclear extracts were prepared from cells treated with 50 μM linoleic acid for 2 h (lanes 2-4) and incubated with anti-p50 antibody (lane 3) or anti-p65 antibody (lane 4) for 25 min before the addition of ^{32}P-labeled probe. Lane 1, probe alone. SS indicate the bands shifted by specific antibodies.

Figure 2. Pretreatment with aspirin, sodium salicylate or PDTC blocks linoleic acid (LA)-induced NF-κB DNA-binding activity in human microvascular endothelial cells (HMEC-1) as measured by EMSA. HMEC-1 were pretreated for 1 h with indicated concentrations of (A) aspirin, (B) sodium salicylate, or (C) for 30 min with PDTC, before a 2 h treatment with 50 μM of linoleic acid (lanes 4-6). Lane 1, probe alone; lane 2, treatment with 50 μM linoleic acid alone; lane 7, competition study performed by the addition of excess unlabeled oligonucleotide using nuclear extract from cells treated with 50 μM linoleic acid.

Figure 3. Time- and dose-dependent upregulation of the VCAM-1 mRNA expression in human microvascular endothelial cells (HMEC-1) induced by linoleic acid as measured by RT-PCR. HMEC-1 were exposed to (A) 50 μM of linoleic acid for the indicated period of time, or (B) treated with increasing concentrations of linoleic acid for 4 h. PCR products were analyzed by 2% agarose gel electrophoresis and visualized using phosphoimaging. The predicted sizes of

RT-PCR products for VCAM-1 and β-actin are 260 bp and 188 bp, respectively. M, molecular weight markers (100-bp DNA ladder).

Figure 4. Linoleic acid increases VCAM-1 protein expression in human microvascular endothelial cells (HMEC-1) as measured by flow cytometry. HMEC-1 were exposed to increasing concentrations of linoleic acid for 12 h. Data are mean ± SD, expressed as relative fluorescence intensity and corrected for unspecific binding. *Values in the group treated with linoleic acid are statistically significant as compared to the untreated control ($P<0.05$).

Figure 5. Pretreatment with aspirin, sodium salicylate or PDTC impedes the induction of VCAM-1 mRNA expression in linoleic acid-treated human microvascular endothelial cells (HMEC-1). Cells were pretreated for 1 h with indicated concentrations of (A) aspirin, (B) sodium salicylate, or (C) for 30 min with PDTC, before a 4 h treatment with 50 μM of linoleic acid and assayed for VCAM-1 mRNA expression by RT-PCR. LPS (1 μg/mL) was used as positive control.

Figure 6. Functional analysis of the NF-κB binding site of the human VCAM-1 promoter in linoleic acid-treated human microvascular endothelial cells (HMEC-1). Cells were transfected with the pF3-CAT3 or the pF3-mNF-κB-CAT3 construct and either untreated or treated with linoleic acid (50 μM) for 24 h. Mutation of the NF-κB site in the VCAM-1 promoter construct completely inhibited linoleic acid-induced CAT activity. *Values in the group treated with linoleic acid are statistically different as compared to the untreated control ($P<0.05$).

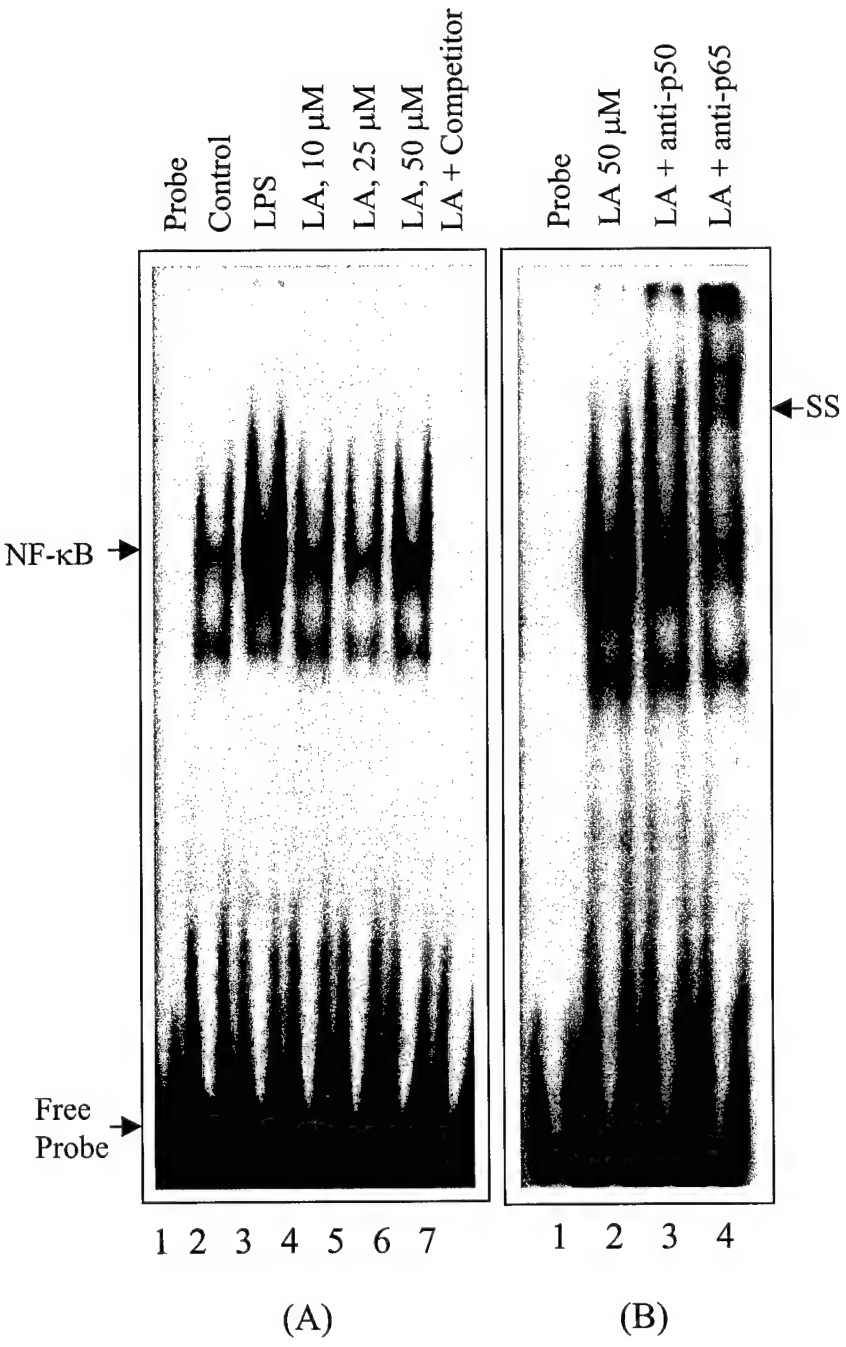

Probe
Control
LPS
LA, 10 μM
LA, 25 μM
LA, 50 μM
LA + Competitor
NF-κB
Free Probe
1 2 3 4 5 6 7
(A)
Probe
LA 50 μM
LA + anti-p50
LA + anti-p65
SS
1 2 3 4
(B)

Figure 1

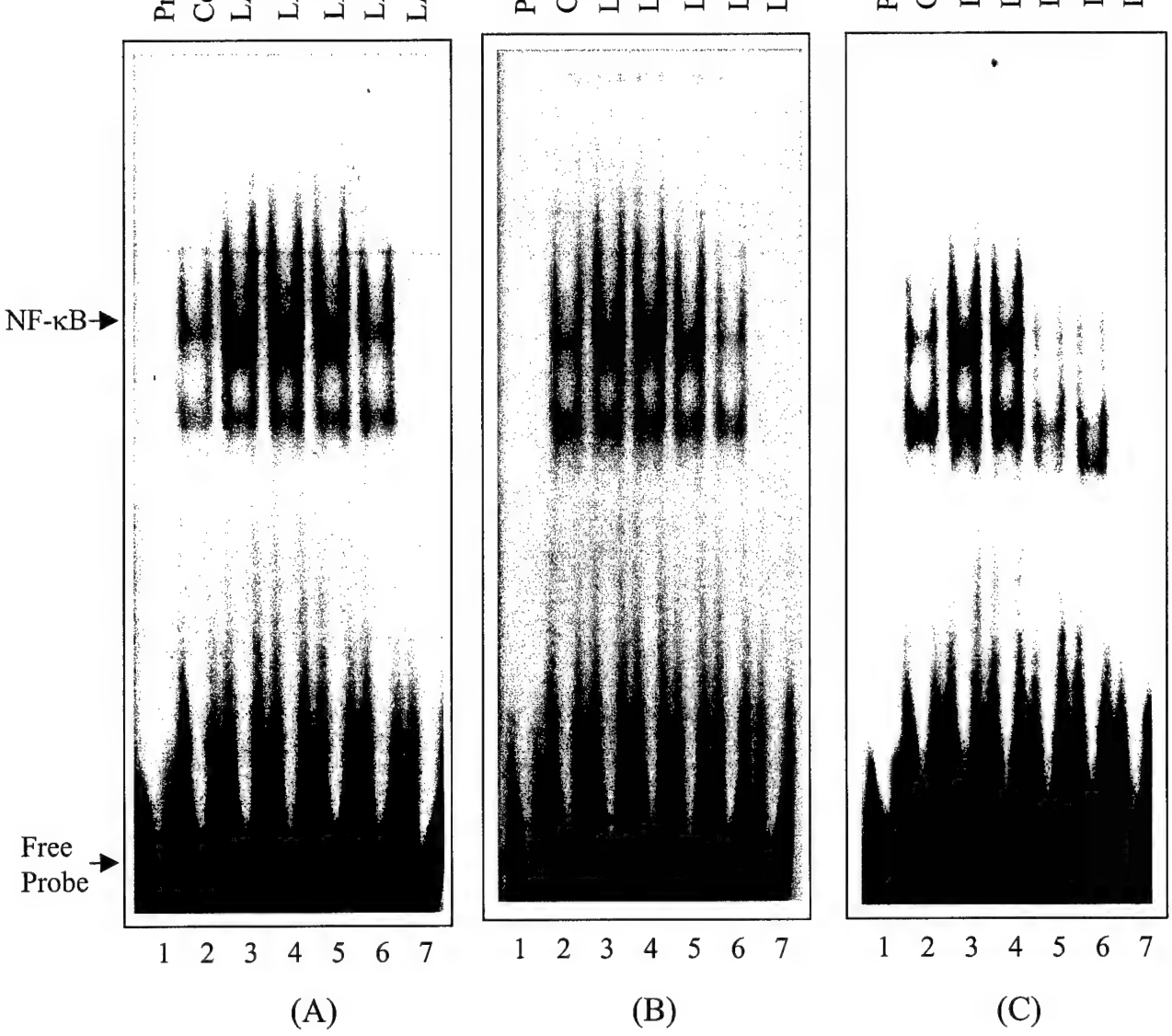
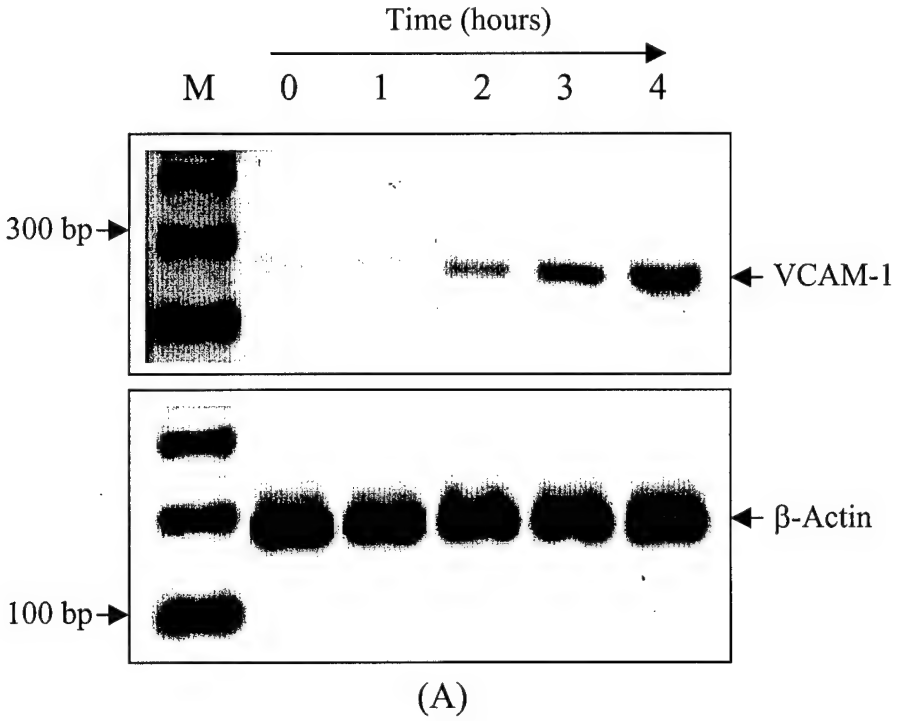
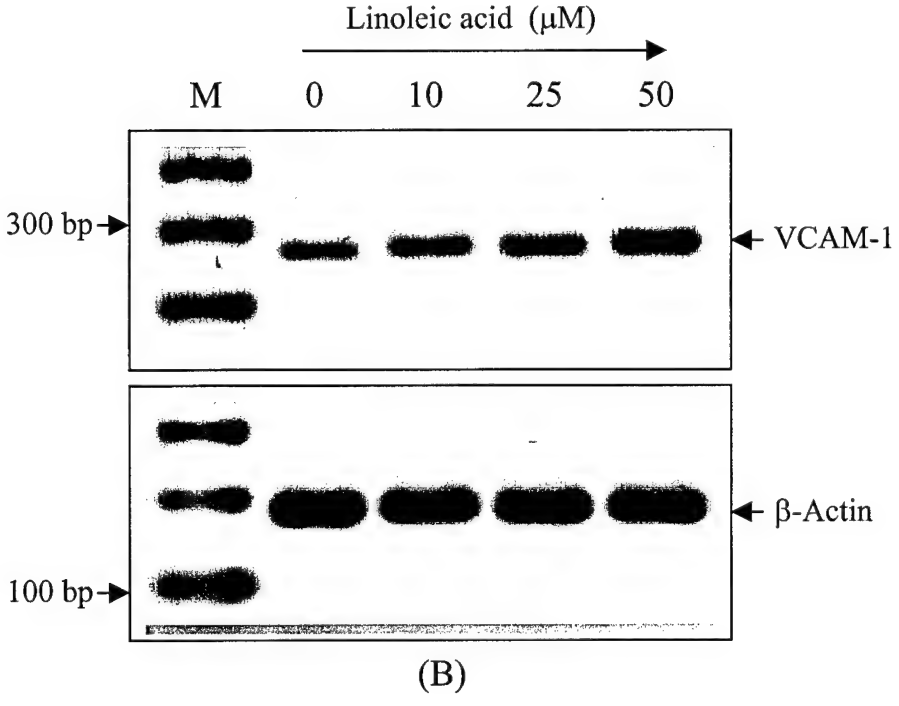

Probe
Control
LA
LA + Aspirin, 2 mM
LA + Aspirin, 5 mM
LA + Aspirin, 10 mM
LA + Competitor
NF-κB
Free Probe
1 2 3 4 5 6 7
(A)
Probe
Control
LA
LA + Na-salicylate, 1 mM
LA + Na-salicylate, 5 mM
LA + Na-salicylate, 10 mM
LA + Competitor
1 2 3 4 5 6 7
(B)
Probe
Control
LA 50 μM
LA + PDTC, 1 μM
LA + PDTC, 10 μM
LA + PDTC, 25 μM
LA + Competitor
1 2 3 4 5 6 7
(C)

Figure 2


Time (hours)
M
0
1
2
3
4
300 bp
VCAM-1
β-Actin
100 bp

(A)


Linoleic acid (μM)
M
0
10
25
50
300 bp
VCAM-1
β-Actin
100 bp

(B)

Figure 3

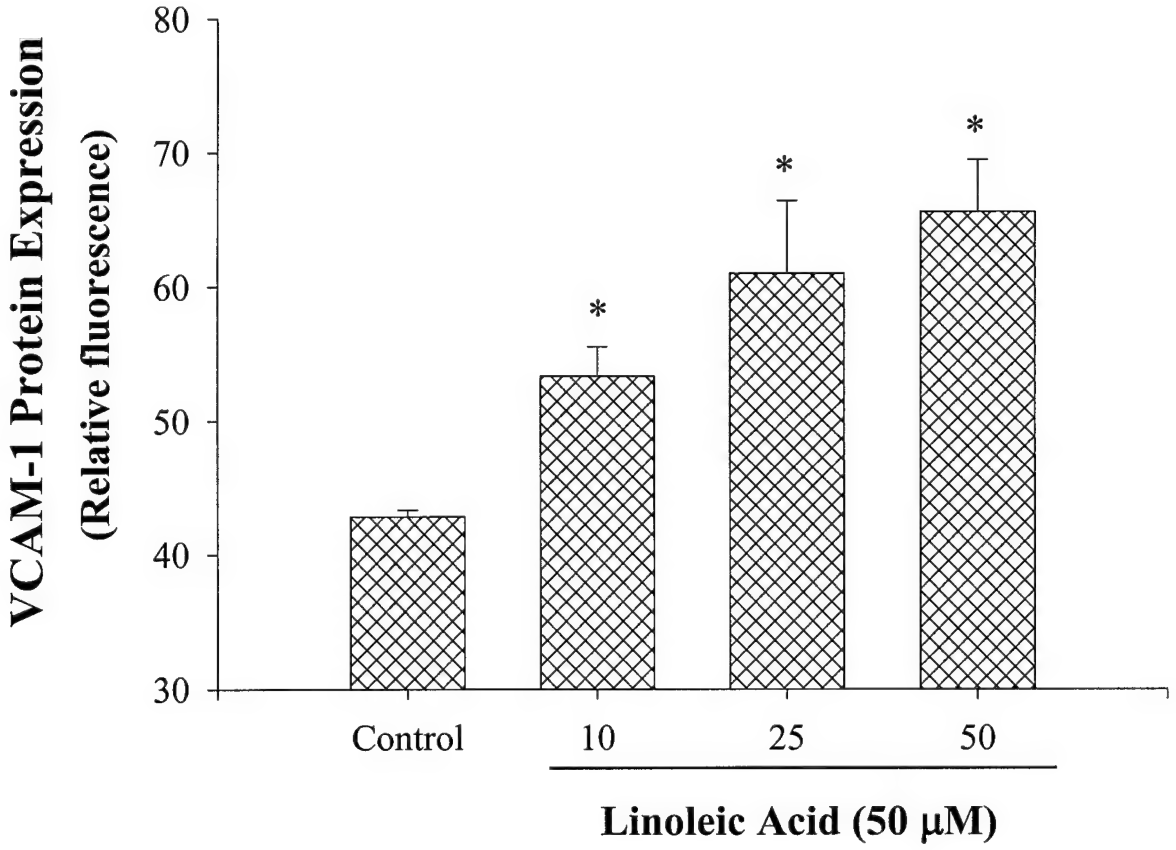
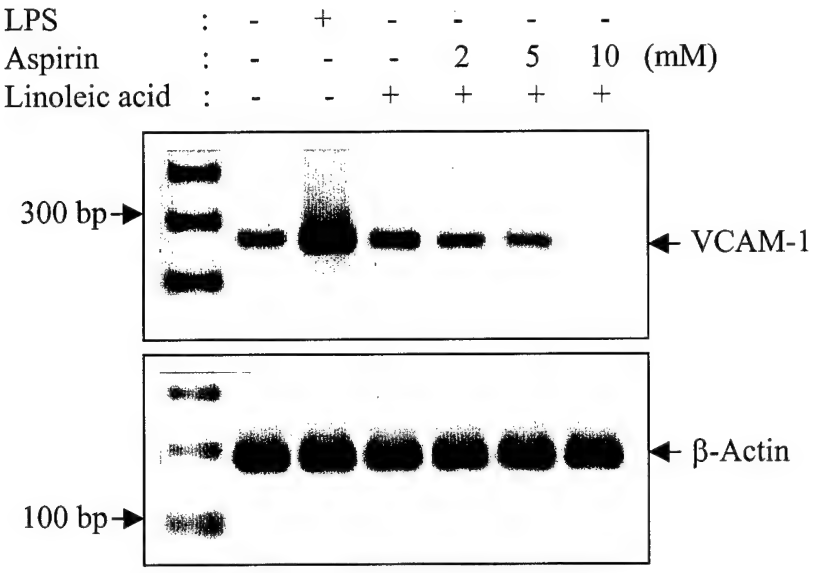
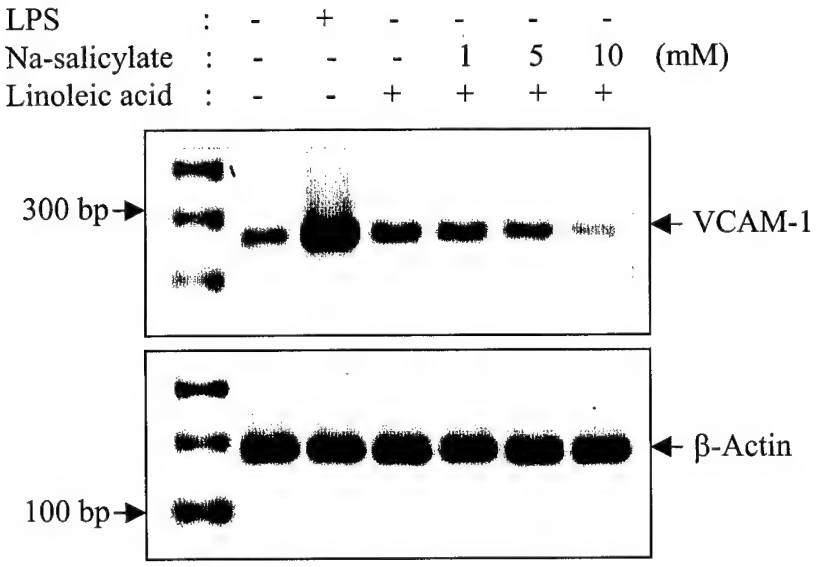

VCAM-1 Protein Expression
(Relative fluorescence)
80
70
60
50
40
30
*
*
*
Control
10
25
50
Linoleic Acid (50 μM)

Figure 4

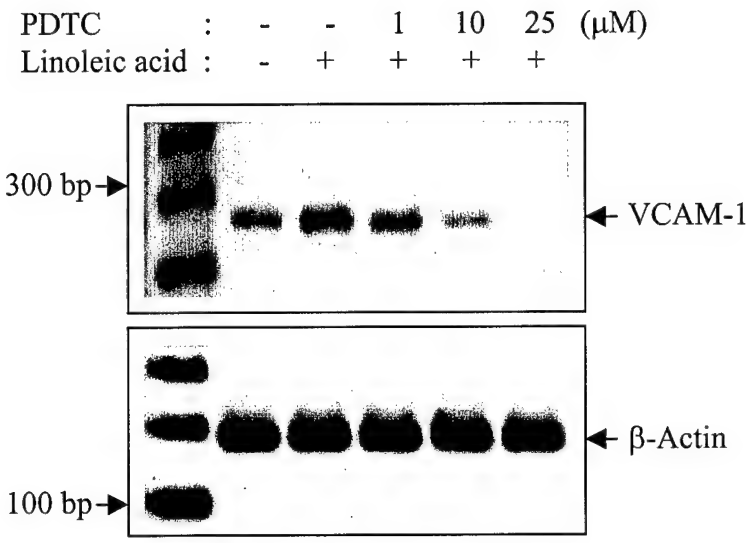


(A)



(B)

Figures 5A and 5B



(C)

Figure 5C

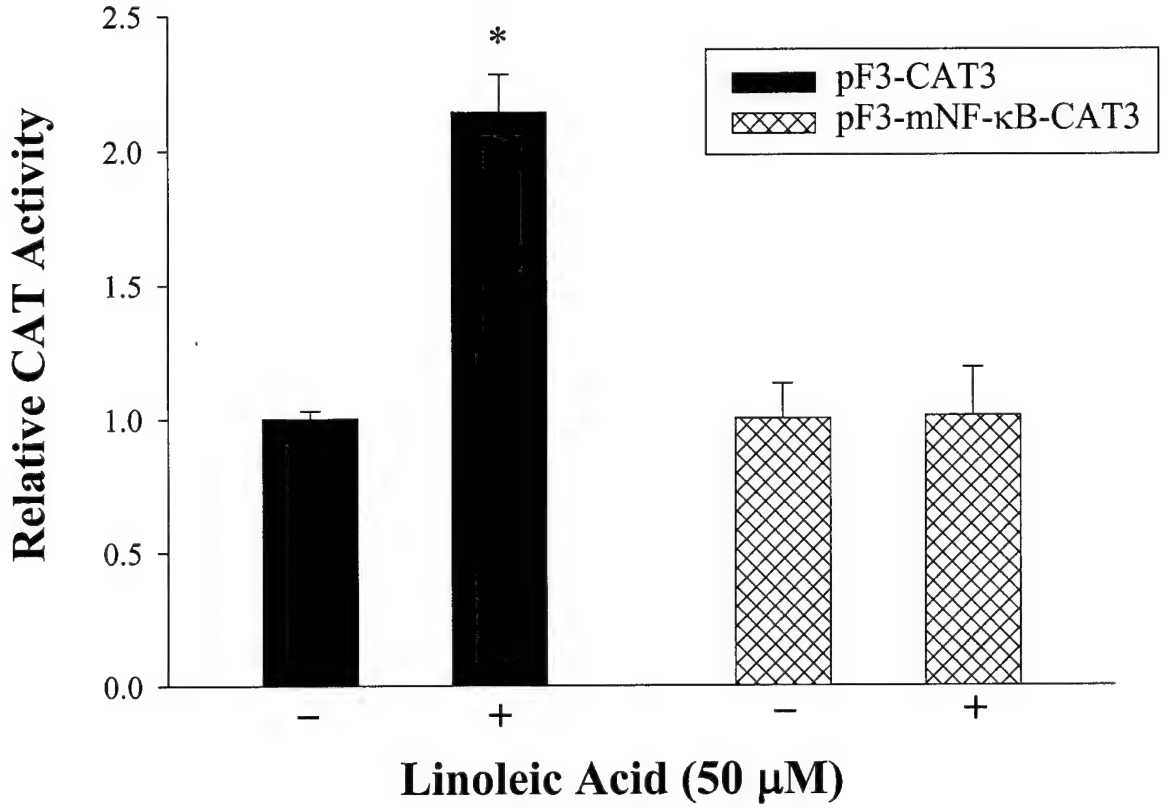

2.5
2.0
1.5
1.0
0.5
0.0
*
pF3-CAT3
pF3-mNF-κB-CAT3
Relative CAT Activity
–
+
–
+
Linoleic Acid (50 μM)

Figure 6

Unsaturated fatty acids selectively induce an inflammatory environment in human endothelial cells

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Abbreviations: 13-HODE, 13-hydroxyoctadecadienoic acid; 13-HPODE, hydroperoxyoctadecadienoic acid; AP-1, activator protein-1; HUVEC, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; NF-κB, nuclear factor-κB; PHGPx, phospholipid hydroperoxide glutathione peroxidase; RT-PCR, reverse transcriptase-polymerase chain reaction; TNF-α, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1.

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Running title: Fatty acids and inflammatory responses

ABSTRACT

Background: Activation of the vascular endothelium by dietary fatty acids may be among the most critical early events in the development of atherosclerosis. However, the specific effects of fatty acids on endothelial cell inflammatory response are not fully understood.

Objective: The present study focused on the induction of inflammatory genes in human endothelial cells exposed to individual dietary fatty acids. Because of the significance of NF-κB and AP-1 in the regulation of inflammatory gene expression, the effects of fatty acids on NF-κB and AP-1 transcriptional activation were also determined.

Design: Human umbilical cord endothelial cells (HUVEC) were exposed to dietary mono- and polyunsaturated 18-carbon fatty acids. Transcriptional activation of NF-κB and AP-1 was determined in HUVEC transfected with reporter constructs regulated by these transcription factors. Induction of the inflammatory genes was studied using reverse transcriptase-polymerase chain reaction (RT-PCR).

Results: Linoleic acid stimulated NF-κB and AP-1 transcriptional activation to the highest extent. In addition, treatment with this fatty acid markedly enhanced mRNA levels of TNF-α, MCP-1, VCAM-1 or ICAM-1. Treatment with linolenic acid stimulated only a moderate induction of these genes, and exposure to oleic acid either had no affect or resulted in decreased inflammatory gene mRNA levels. In addition, exposure to both linoleic acid and linolenic acid strongly stimulated induction of the phospholipid hydroperoxide glutathione peroxidase (PHGPx) gene in HUVEC.

Conclusion: These results demonstrate that specific unsaturated dietary fatty acids, and in particular linoleic acid, can selectively stimulate the development of a proinflammatory environment within the vascular endothelium.

Key Words: fatty acids, inflammatory genes, transcription factors, human endothelial cells, atherosclerosis.

INTRODUCTION

Activation or dysfunction of the vascular endothelium is one of the first events in the development of atherosclerosis (1,2), and selected dietary fatty acids may be among the most critical factors which induce these processes. For example, it has been demonstrated that lipids, including selective free fatty acids, may cause injury to the endothelium (reviewed in [3]). It has been proposed that hydrolysis of triglyceride-rich lipoproteins mediated by lipoprotein lipase, a key enzyme in lipoprotein metabolism that is associated with the luminal site of endothelial cells, may be an important source of high concentrations of fatty acid anions in the proximity to the endothelium (4,5). To support this notion, it has been demonstrated that activity of lipoprotein lipase is increased in atherosclerotic lesions (5-7). Lipoprotein lipase-derived remnants of lipoproteins isolated from hypertriglyceridemic subjects as well as selective unsaturated fatty acids can disrupt endothelial integrity (8,9). Because plasma and tissue lipid composition is closely related to dietary fat intake (10), endothelial cell exposure to individual free fatty acids can be directly influenced by types of fatty acids consumed in the diet (10,11).

Strong evidence indicates that exposure to selected unsaturated dietary 18-carbon fatty acids can directly affect endothelial cell metabolism. Significant amounts of data have been accumulated to demonstrate that linoleic acid (18:2, n-6) can induce a marked injury to endothelial cells. For example, it has been reported that this fatty acid can disrupt endothelial cell integrity, alter functions of gap-junctional proteins (12), increase levels of intracellular calcium, and induce cellular oxidative stress (13). In addition, the treatment of endothelial cells with linoleic acid and tumor necrosis factor-α (TNF-α) can activate caspase 3 activity and induce apoptotic cell death (14,15). The role of other unsaturated dietary 18-carbon fatty acids in endothelial cell metabolism is less understood. However, evidence indicates that dietary oleic acid can protect endothelial cells against hydrogen peroxide-induced oxidative stress (16), as well as reduce the susceptibility of low density lipoproteins to oxidative modifications (17).

Atherosclerosis is recognized to be an inflammatory disease of the vascular wall (18), and endothelial cell inflammatory reactions are primarily regulated by production of chemokines (e.g., monocyte chemoattractant protein-1; MCP-1), inflammatory cytokines (e.g., TNF-α), and adhesion molecules (e.g., intercellular adhesion molecule-1; ICAM-1 and vascular cell adhesion molecule-1; VCAM-1). Expression of these inflammatory mediators and their effects are closely

interrelated. In addition, overexpression of MCP-1 (19), TNF-α (20), ICAM-1 and VCAM-1 (21) is a common feature of atherosclerotic processes.

Inflammatory genes, such as genes encoding for MCP-1, TNF-α, ICAM-1 or VCAM-1, are regulated by a variety of transcription factors (2,22). However, it appears that NF-κB and AP-1 may play a critical role in these regulatory processes. The binding sites for these transcription factors were identified in the promoter regions of a variety of inflammatory genes (22-25), and increased levels of NF-κB were found in atherosclerotic vessels (26,27). In addition, selected free fatty acids, such as linoleic acid, can activate NF-κB in endothelial cells (13). Moreover, effects mediated by NF-κB and AP-1 appear to be interrelated. For example, it was shown that TNF-α-mediated induction of VCAM-1 expression requires both activated NF-κB and AP-1 (23).

Although it is known that selected free fatty acids can induce oxidative stress and activate oxidative stress responsive transcription factors (13), the specific effects of unsaturated fatty acids on endothelial cell inflammatory response are not fully understood. Therefore, the focus of the present study is to examine the induction of the inflammatory genes in human endothelial cells exposed to specific 18-carbon, mono- and polyunsaturated fatty acids. In addition, due to of the significance of NF-κB and AP-1 in the regulation of the inflammatory genes, the effects of unsaturated fatty acids on the transcriptional activity of these transcription factors were also determined.

MATERIAL AND METHODS

Human umbilical vein endothelial cell (HUVEC) cultures and fatty acid treatments

Human umbilical vein endothelial cells (HUVEC) were isolated as described previously (28) and cultured in enriched M199 medium, which included 25 mmol/L HEPES, 54.3 U/mL heparin, 2 mmol/L L-glutamine, 1 μmol/L sodium pyruvate, 200 U/mL penicillin, 200 μg/mL streptomycin, 0.25 μg/mL amphotericin B (GibcoBRL, Grand Island, NY), 0.04 mg/mL endothelial cell growth supplement (ECGS, Becton Dickinson, Bedford, MA), and 20% FBS (HyClone, Logan, UT). Cells were determined to be endothelial in origin by their cobblestone morphology and uptake of fluorescent labeled acetylated low-density lipoprotein (1,1'-

dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; Molecular Probes, Eugene, OR). All experiments were performed on cells from passage 2. Confluent cell cultures were treated with up to 180 μmol/L of oleic acid (18:1, n-9), linoleic acid (18:2, n-6) or linolenic acid (18:3, n-3) (Nu-Chek Prep, Elysian, MN). Fatty acid-enriched experimental media were prepared as described earlier (9).

Transfections and reporter gene assay

Transfections were performed as described earlier (29). Briefly, HUVEC were seeded in 12-well plates and grown to 50-60% confluency in normal growth medium. Then, aliquots of normal M199 were mixed with 36 μg/mL of a liposome pFx-7 (Invitrogen, Carlsbad, CA) and with 10 μg/mL of NF-κB- or AP-1-responsive plasmids (pNFκB-Luc or pAP1-Luc, respectively), containing luciferase reporter gene (Stratagene, La Jolla, CA). The transfection mixtures were incubated at 37°C for 30 min to allow formation of DNA-lipid complexes. Endothelial cell cultures were washed with M199 to remove serum, and 1 mL of transfection solution was added for 1.5 h to each well of the 12 well plate. After incubation, transfection solutions were aspirated and replaced with growth medium for 24 h. Then, transfected cultures were treated with specific unsaturated fatty acids for 24 h. Control groups consisted of transfected HUVEC cultures which were not exposed to fatty acids.

Luciferase activity was measured by Luciferase Assay System (Promega). Briefly, culture media were removed, HUVEC were washed with PBS and incubated with Cell Culture Lysis reagent. Cell lysates were centrifuged to remove membrane debris and 10 μL of the cell extracts were mixed with 100 μL of Luciferase Assay Reagent containing luciferin and ATP in a luminometer with automatic injection. Values are expressed in RLU/μg protein.

Transfection efficiency was monitored as described earlier (29) by transfection of endothelial cells with the VR-3301 vector which contains human placental alkaline phosphatase (hpAP) as the reporter gene. Under the described conditions, transfection efficiency was determined to be 32% (29). All transfection studies were repeated three times using 6 wells in 12-well plates per experimental group.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analyses

RT-PCR reactions were performed as described earlier (28,30). Briefly, treated HUVEC were lysed and the total RNA was extracted using RNA STAT-60 (Tel-TEST, Inc.) according to the procedure supplied by the manufacturer. Isolated RNA was quantitated by determining absorbance at 260 nm. A standard reverse transcription (RT) reaction was performed at 42 °C for 60 min in 20 μL of 5 mmol/L $MgCl_2$, 10 mmol/L Tris-Cl, pH 9.0, 50 mmol/L KCl, 0.1% Triton X-100, 1 mmol/L dNTP, 1 unit/μL of recombinant RNasin ribonuclease inhibitor, 15 unit/μg of AMV reverse transcriptase, and 0.5 μg of oligo$(dT)_{15}$ primer (Promega, Madison, WI). Table 1 reflects sequences of the primer pairs that were used for PCR amplification of the studied genes. For quantitation, levels of mRNA of the studied inflammatory genes and the PHGPx gene was related to the β-actin mRNA (a housekeeping gene). The PCR mixture consisted of 2 μL of a product of the RT reaction, a Taq PCR Master Mix Kit (Qiagen, Valencia, CA) and 20 pmol of primer pairs in a total volume of 50 μL. For each individual gene, a linear range of PCR amplification was established and the induction of the target gene was studied within such a range. The numbers of cycles and thermocycling conditions employed in PCR analyses of inflammatory gene mRNA levels are given in Table 1. Under the described RT-PCR conditions, the β-actin transcript (a housekeeping gene) increases linearly in the range of 15-40 PCR cycles. Therefore, induction of the β-actin gene was determined using the same number of cycles as for the target genes. PCR products were separated by 2% agarose gel electrophoresis, stained with SYBR® Green I (Molecular Probes, Eugene, OR) and visualized using phosphoimaging technology (FLA-2000, Fuji, Stamford, CT). Relative intensity of fluorescence (ratio of the intensity of the band corresponding to the target gene compared to that corresponding to the β-actin gene) was quantified using Image Gauge 3.0 software (Fuji) and expressed in average pixel intensity. Experiments were repeated four times and the values of relative fluorescence were subjected to statistical analysis.

Statistical analysis

Statistical analysis was performed using SYSTAT 8.0 (SPPS Inc., Chicago, IL). One-way or two-way ANOVA were used to compare the mean values among the treatments. When the overall F values were significant, ANOVA analysis was followed by posthoc Bonferroni test

to compare means from different treatments. Statistical probability of $p<0.05$ was considered significant.

RESULTS

To study the dose-dependent effects of specific unsaturated fatty acids on the mRNA levels related to the genes critical for endothelial cell inflammatory response, HUVEC were exposed to 60, 90, and 180 μmol/L of free fatty acids. Preliminary experiments revealed that free fatty acids consistently exerted a maximum effect on the inflammatory gene induction at the concentration of 90 μmol/L. Therefore, experiments with 180 μmol/L of fatty acids were discontinued and data are presented from the studies in which HUVEC were exposed only to 60 and 90 μmol/L of unsaturated free fatty acids.

Unsaturated fatty acids selectively induce NF-κB and AP-1 transcriptional activation

Oxidative stress may affect cellular metabolism by activation of oxidative stress-responsive transcription factors, such as NF-κB and AP-1. Therefore, the effects of unsaturated fatty acids on NF-κB or AP-1 transcriptional activation were determined. HUVEC were transfected with luciferase reporter plasmids, the expression of which was regulated either by NF-κB or by AP-1. Then, transfected cells were treated with specific unsaturated fatty acids and luciferase activity was measured as a marker of either NF-κB or AP-1 transcriptional activation (Figure 1).

Figure 1A reflects the effects of specific unsaturated fatty acids on NF-κB transcriptional activation. Treatment of endothelial cells with oleic acid did not affect luciferase activity in cells transfected with NF-κB-responsive reporter plasmid (pNFκB-Luc). As compared to control cultures, linolenic acid exerted only a moderate effect on NF-κB transcriptional activation; however, treatment of transfected endothelial cells with linoleic acid resulted in a most pronounced increase in luciferase activity, indicating a marked increase in transcriptional activation of NF-κB .

Similar results were observed in endothelial cells transfected with a reporter plasmid responsive to AP-1 (pAP1-Luc) and treated with selected unsaturated fatty acids (Figure 1B). Among the fatty acids tested, linoleic acid stimulated AP-1 transcriptional activation most markedly, as compared to control cultures. In contrast, linolenic acid exerted more moderate effects, and oleic acid did not affect luciferase expression dependent on transcriptional activation of AP-1.

Unsaturated fatty acids selectively induce the MCP-1 and TNF-α genes

MCP-1 and TNF-α are critical inflammatory mediators of inflammatory reactions involved primarily in leukocyte recruitment and upregulation of expression of adhesion molecules and other inflammatory cytokines. The effects of treatment with selected unsaturated fatty acids on MCP-1 mRNA levels are shown in Figure 2A. Among tested fatty acids, linoleic acid at the concentration of 90 μmol/L stimulated the most pronounced induction of the MCP-1 gene (51% over the control values as measured by the density of the fluorescent bands). Indeed, MCP-1 mRNA levels in endothelial cells treated with 90 μM of linoleic acid for 3 h were in the range of that observed in cells exposed to 20 ng/mL of TNF-α, which was used as a positive control. Levels of MCP-1 mRNA levels were also increased in endothelial cells treated with 60 and 90 μmol/L linolenic acid (by 24% and 30%, respectively). In contrast, induction of the MCP-1 gene in endothelial cells exposed to oleic acid was approximately at the range observed in non-stimulated endothelial cells.

Figure 2B depicts the effects of selected unsaturated fatty acids on TNF-α mRNA levels, as measured by RT-PCR. Similar to the results on the MCP-1 gene induction, treatment of HUVEC with linoleic acid markedly induced TNF-α mRNA levels (21% over the control values). In addition, linolenic acid at the dose of 90 μmol/L also stimulated similar induction of the TNF-α gene. Independent of the dose, treatment with oleic acid did not affect TNF-α mRNA levels in cultured HUVEC.

Unsaturated fatty acids induce selective induction of the genes encoding for adhesion molecules

Among adhesion molecules, ICAM-1 and VCAM-1 play an important role in mediating the firm adhesion of leukocytes to the surface of endothelial cells. The effects of treatment with selected unsaturated fatty acids on ICAM-1 mRNA levels is shown in Figure 2C. Exposure to both linoleic acid and linolenic acid induced a dose dependent increase in ICAM-1 mRNA levels. In addition, these two fatty acids at the concentration of 90 μmol/L stimulated induction of the ICAM-1 gene to a similar extent, i.e., by approximately 30% as measured by the density of the appropriate fluorescent bands. In contrast, exposure of HUVEC to oleic acid decreased ICAM-1 mRNA levels to approximately 50% of control values.

The effects of specific unsaturated fatty acids on VCAM-1 mRNA levels in HUVEC are indicated in Figure 2D. The most significant induction of the VCAM-1 gene (by 38% as measured by the density of the fluorescent bands) was observed in cells treated with 90 μmol/L of linoleic acid. In addition, exposure to 90 μmol/L of linolenic acid resulted in a slight increase in VCAM-1 mRNA levels. Treatment with oleic acid had no effect on VCAM-1 gene induction as compared to control cultures.

Unsaturated fatty acids induce PHGPx mRNA levels

PHGPx is an antioxidant enzyme that is involved in detoxification of lipid hydroperoxides in cellular membranes and lipoproteins (31). Thus, this enzyme may play a critical role in antioxidant protection against unsaturated fatty acid-induced oxidative stress. The effects of selected fatty acids on PHGPx mRNA levels in HUVEC are shown in Figure 3. As compared to control cells, treatment with oleic acid increased PHGPx mRNA levels by approximately 30%. However, both linoleic acid and linolenic acid strongly, and in a dose-dependent manner, stimulated induction of the PHGPx gene in HUVEC. In fact, treatment with 60 and 90 μmol/L linoleic acid enhanced the PHGPx mRNA levels by 60% and 104%, respectively. Furthermore, exposure to 60 and 90 μmol/L linolenic acid increased induction of the PHGPx gene by 108 and 121%, respectively.

DISCUSSION

Mono- and polyunsaturated 18-carbon fatty acids provide a unique model to study the cellular effects of fatty acids that differ in unsaturation but independent of carbon length (9). In addition, unsaturated fatty acids employed in the present study are major dietary fatty acids. Endothelial cells were exposed to these fatty acids at concentrations of 60 or 90 μmol/L, with albumin concentration in the experimental media to be 60 μmol/L. Normal plasma free fatty acid concentrations can range from approximately 90-1200 μM; however, the majority of free fatty acids are bound to plasma components, mostly albumin (32,33). In fact, the main factor in the availability of fatty acids for cellular uptake is determined by the free fatty acid:albumin ratio. Normally, this ratio can range from 0.15 to 4 under various conditions, with an average of approximately 1 (32,33). Thus, the experimental conditions employed in the present study, which resulted in a free fatty acid:albumin ratio of 1 or 1.5, were within physiologic range.

One of the most important functions of the vascular endothelium is regulation of inflammatory reactions (1). The development of inflammatory reactions is a normal defense mechanism in response to injury or activation of the vessel wall. The physiologic significance of such reactions is to maintain and repair normal structure and function of the vessel wall. However, excessive inflammatory reactions with the development of a positive feedback inflammatory cycle can lead to severe tissue damage, and it is associated with vascular pathology, including the development of the atherosclerotic plaques (34). Induction of genes encoding for mediators of inflammatory response, i.e., inflammatory cytokines, chemokines, and adhesion molecules, can initiate leukocyte infiltration of the vessel wall. These mediators of inflammatory responses very closely interact with each other in *in vivo* conditions. For example, ICAM-1 and VCAM-1 facilitate leukocyte adhesion to the vascular endothelium and both MCP-1 (35) and, to a lesser extent, TNF-α (36,37) are potent chemoattractive factors, which play a significant role in recruiting lymphocytes and monocytes into the vessel wall. In addition, TNF-α is a strong inducer of inflammatory reactions and can stimulate overexpression of MCP-1, inflammatory cytokines, as well as ICAM-1 and VCAM-1 (38). In fact, these strong pro-inflammatory properties of TNF-α were the reason that this cytokine was used as the positive control in our present studies on the effects of free fatty acids on induction of inflammatory gene mRNA levels. In addition, the inflammatory genes studied in the present paper, i.e., genes

encoding for VCAM-1, ICAM-1, TNF-α, and MCP-1, are regulated by similar transcription factors, with dominant roles of NF-κB and AP-1 (22-25).

The importance of NF-κB and AP-1 in the induction of inflammatory reactions prompted us to study the effects of specific fatty acids on the transcriptional activity of these transcription factors in human endothelial cells. Among the unsaturated fatty acids studied, linoleic acid induced both NF-κB and AP-1 transcriptional activation most markedly. These data are in agreement with our previous results in which, using electrophoretic mobility shift assay, we demonstrated a marked activation of NF-κB (13) and AP-1 (39) in endothelial cells exposed to linoleic acid. It is possible that fatty acid-induced endothelial cell oxidative stress and disturbances in the glutathione redox status could be responsible for activation of these oxidative stress-responsive transcription factors. Intercellular glutathione is the major non-protein thiol compound which regulates the cellular redox status. It was demonstrated that depletion of glutathione levels and alterations of equilibrium between its reduced and oxidized derivatives can stimulate activation of NF-κB (40). To support this notion, we demonstrated that exposure of endothelial cells to unsaturated fatty acids can result in a marked decrease in cellular glutathione levels and activation of NF-κB (9,13). In addition, the glutathione precursor N-acetylcysteine, prevented fatty acid-induced activation of NF-κB (41).

Glutathione peroxidases are a family of antioxidant enzymes that utilize glutathione in the reduction of hydrogen peroxide and alkyl hydroperoxides. Among a variety of glutathione peroxidases, PHGPx plays a unique role. In addition to reducing hydrogen peroxide and soluble hydroperoxides, PHGPx is the only antioxidant enzyme that can reduce hydroperoxy fatty acids which are integrated in cellular membranes (42) or lipoproteins (43). PHGPx was also shown to be involved in silencing activities of cyclooxygenase or 5- and 15-lipoxygenases (44,45), enzymes involved in metabolism of unsaturated fatty acids. Results of the present study demonstrate that exposure of endothelial cells to specific unsaturated fatty acids can markedly stimulate induction of PHGPx mRNA levels. In addition, the fatty acid-stimulated increases in PHGPx mRNA levels appeared to be correlated with the amount of unsaturated bonds in fatty acid molecules. For example, linolenic acid, followed by linoleic acid, enhanced induction of the PHGPx gene most markedly.

The present study provides compelling evidence that linoleic acid can induce profound inflammatory responses in cultured human endothelial cells. In fact, among all of the

unsaturated fatty acids studied, linoleic acid stimulated induction of inflammatory gene mRNA levels most markedly. Because expression of the inflammatory genes is primarily regulated by NF-κB and AP-1, a strong induction of NF-κB and AP-1 transcriptional activation by linoleic acid can explain a marked indication of the studied genes encoding for inflammatory mediators. In addition, not only linoleic acid but also specific oxidative products of this fatty acid can exert proinflammatory effects (46,47). However, we observed that the lipoxygenase metabolites of linoleic acids, such as 13-hydroperoxyoctadecadienoic acid (13-HPODE) or 13-hydroxyoctadecadienoic acid (13-HODE), can induce a different pattern of inflammatory responses in endothelial cells as compared to free linoleic acid. Specifically, exposure of HUVEC to 13-HPODE or 13-HODE did not induce expression of VCAM-1 or E-selectin (48). In addition, polyunsaturated fatty acids, such as linoleic acid, can be non-enzymatically converted to 4-hydroxynonenal (HNE). However, exposure of HUVEC to HNE markedly stimulated apoptosis of vascular endothelial cells but did not result in activation of NF-κB or induction of adhesion molecules (49). Thus, even though linoleic acid can be converted to oxidized metabolites, it appears unlikely that 13-HPODE, 13-HODE or HNE can contribute significantly to inflammatory reactions induced by this fatty acid. On the other hand, the effects of other metabolites of polyunsaturated fatty acids, e.g., derivatives of the cytochrome-P450 pathway, on inflammatory reactions in human endothelial cells remain yet to be determined. Our recent data suggest that epoxide metabolites of linoleic acid may have proinflammatory properties (50).

Although our data clearly indicate that specific unsaturated fatty acids can induce proinflammatory effects in endothelial cells, opposite results were reported when cells were exposed to selected n-3 or n-6 fatty acids for up to 72 h and co-exposed to inflammatory cytokines, such as IL-1β or TNF-α for an additional 12 h. Using such experimental approaches, it was observed that pre-exposure to fatty acids inhibited cytokine-induced expression of inflammatory mediators, such as VCAM-1, on the surface of endothelial cells (51). Similar inhibition of ICAM-1 expression was also observed in cells pretreated with 13-HPODE before stimulation with IL-1β. However, simultaneous administration of 13-HPODE with IL-1β or TNF-α resulted in additive effects on ICAM-1 production (48). We have shown that pre-exposure of endothelial cells to linoleic acid can cross-amplify TNF-α-mediated induction of cellular oxidative stress and endothelial cell dysfunction (13), but it did not potentiate or even

inhibit NF-κB-dependent transcription (13,41). To explain this phenomenon, it was proposed that fatty acid-induced activation of NF-κB could lead to increased levels of NF-κB inhibitory subunit(s), which, in turn, can prevent further activation of this transcription factor in cells exposed to cytokines at later time points (13).

In contrast to linoleic acid and linolenic acid, which exerted strong or moderate proinflammatory responses, respectively, oleic acid diminished inflammatory gene mRNA levels in endothelial cells. These data are in agreement with previous reports on oleic acid-mediated antioxidant effects. For example, a diet enriched in oleic acid markedly decreased LDL susceptibility to oxidation and LDL-protein modification in mildly hypercholesterolemic patients (10). Similar results were also obtained in experimental animals fed an oleic acid-enriched diet (52). Extensive evidence also indicates the protective and antioxidant effects of oleic acid on endothelial cell activation. It was demonstrated that cellular treatment with this fatty acid protected endothelial cells against cytokine-induced VCAM-1, ICAM-1 or E-selectin overexpression (53). In addition, supplementation with oleic acid protected endothelial cells against hydrogen peroxide-induced cytotoxicity (16) as well as against oxidized LDL-mediated endothelial barrier dysfunction (54).

In conclusion, the present study demonstrates that specific unsaturated dietary fatty acids can induce highly individual effects on endothelial cell activation and contribute differently to the induction of the inflammatory genes in vascular endothelial cells. Among the fatty acids studied, linoleic acid stimulated inflammatory gene mRNA levels most markedly. In contrast, oleic acid appeared to silence the induction of a variety of the proinflammatory genes in endothelial cells. These results demonstrate that specific unsaturated dietary fatty acids, such as linoleic acid and, to a lesser extent, linolenic acid, can stimulate the development of proinflammatory environments within the vascular endothelium.

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Table 1. Sequences of the primer pairs employed in the RT-PCR reactions and thermocycling conditions.

Studied gene	Sequences of the primer pairs (5' - 3') and thermocycling conditions
MCP-1*	Forward: CAG CCA GAT GCA ATC AAT GC Reverse: GTG GTC CAT GGA ATC CTG AA Thermocycling: 94 °C for 4 min; followed by 94 °C for 45 s, 55 °C for 45 s, 72 °C for 45 s (repeated 25 times); followed by an extension at 72 °C for 10 minutes
TNF-α*	Forward: AGC CTC TTC TCC TTC CTG AT Reverse: AGT AGA TGA GGG TCC AGG AG Thermocycling: 94 °C for 4 min; followed by 94 °C for 45 s, 55 °C for 45 s, 72 °C for 45 s (repeated 25 times); followed by an extension at 72 °C for 10 minutes
ICAM-1	Forward: GGT GAC GCT GAA TGG GGT TCC Reverse: GTC CTC ATG GTG GGG CTA TGT CTC Thermocycling: 94 °C for 4 min; followed by 94 °C for 45 s, 60 °C for 45 s, 72 °C for 60 s (repeated 28 times); followed by an extension at 72 °C for 7 minutes
VCAM-1*	Forward: ATG ACA TGC TTG AGC CAG G Reverse: GTG TCT CCT TCT TTG ACA CT Thermocycling: 94 °C for 60 s, 55 °C for 60 s, 72 °C for 60 s (repeated 25 times)
PHGPx	Forward: TGT GCG CGC TCC ATG CAC GAG T Reverse: AAA TAG TGG GGC AGG TCC TTC TCT Thermocycling: 94 °C for 4 min; followed by 94 °C for 40 s, 66 °C for 60 s, 72 °C for 2 min (repeated 20 times); followed by an extension at 72 °C for 7 minutes
β-Actin†	Forward: AGC ACA ATG AAG ATC AAG AT Reverse: TGT AAC GCA ACT AAG TCA TA

* Primer pairs purchased from R&D Systems (Minneapolis, MN). † A housekeeping gene, induction of the β-actin gene was determined using the same number of cycles and thermocycling conditions as for the target genes. Abbreviations: ICAM-1, intercellular adhesion molecule-1; monocyte chemoattractant protein-1; PHGPx, phospholipid hydroperoxide glutathione peroxidase; TNF-α, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1.

FIGURE LEGENDS

Figure 1A. Fatty acid-induced NF-κB-related transcription in human endothelial cells. Transcriptional activation of NF-κB was measured by luciferase activity in HUVEC transfected with NF-κB-responsive luciferase reporter construct and exposed to specific unsaturated fatty acids (90 μmol/L) for 24 h. TNF-α treatment was used as a positive control. Data are mean ± SEM, n c 6. *Significantly different from control cultures. Abbreviations: 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid; TNF-α, tumor necrosis factor-α (positive control).

Figure 1B. Fatty acid-induced AP-1-related transcription in human endothelial cells. Transcriptional activation of AP-1 was measured by luciferase activity in HUVEC transfected with AP-1-responsive luciferase reporter construct and exposed to specific unsaturated fatty acids (90 μmol/L) for 24 h. TNF-α treatment was used as a positive control. Data are mean ± SEM, n=6. *Significantly different from control cultures. Abbreviations as in the legend to Figure 1A.

Figure 2A. Effects of dietary fatty acids on monocyte chemoattractant protein-1 (MCP-1) mRNA levels in human endothelial cells as measured by RT-PCR. HUVEC were exposed to specific unsaturated fatty acids for 3 hours. β-Actin was used as a housekeeping gene to indicate that the same amount of RNA was used per sample. The amplified PCR products were electrophoresed on a 2% TBE agarose gel, stained with SYBR Green I (Molecular Probes, Eugene, OR) and visualized using phosphoimaging technology (FLA-2000, Fuji, Stamford, CN). Abbreviations as in the legend to Figure 1A.

Figure 2B. Effects of dietary fatty acids on tumor necrosis factor-α (TNF-α) mRNA levels in human endothelial cells as measured by RT-PCR. Experiments were performed as described in the legend to Figure 2A. Abbreviations as in the legend to Figure 1A.

Figure 2C. Effects of dietary fatty acids on intercellular adhesion molecule-1 (ICAM-1) mRNA levels in human endothelial cells as measured by RT-PCR. Experiments were performed as described in the legend to Figure 2A. Abbreviations as in the legend to Figure 1A.

Figure 2D. Effects of dietary fatty acids on vascular cell adhesion molecule-1 (VCAM-1) mRNA levels in human endothelial cells as measured by RT-PCR. Experiments were performed as described in the legend to Figure 2A. Abbreviations as in the legend to Figure 1A.

Figure 3. Effects of dietary fatty acids on phospholipid hydroperoxide glutathione peroxidase (PHGPx) mRNA levels in human endothelial cells as measured by RT-PCR. HUVEC were exposed to specific unsaturated fatty acids for 24 hours. RT-PCR was performed as described in the legend to Figure 2A. Abbreviations as in the legend to Figure 1A.

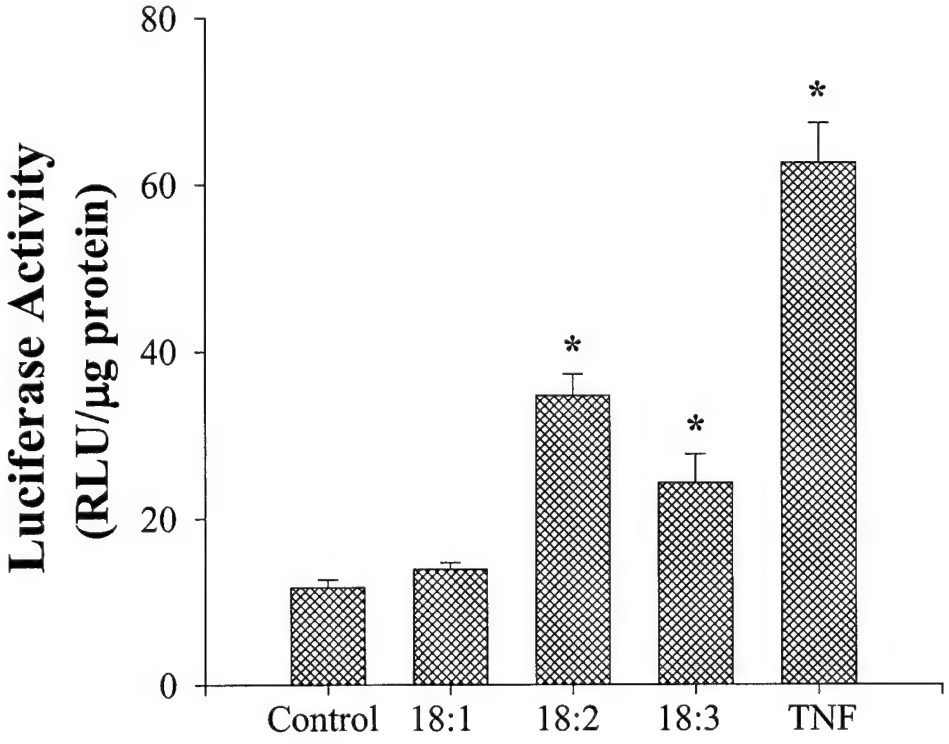
Luciferase Activity
(RLU/µg protein)
80
60
40
20
0
*
*
*
Control
18:1
18:2
18:3
TNF

Figure 1A

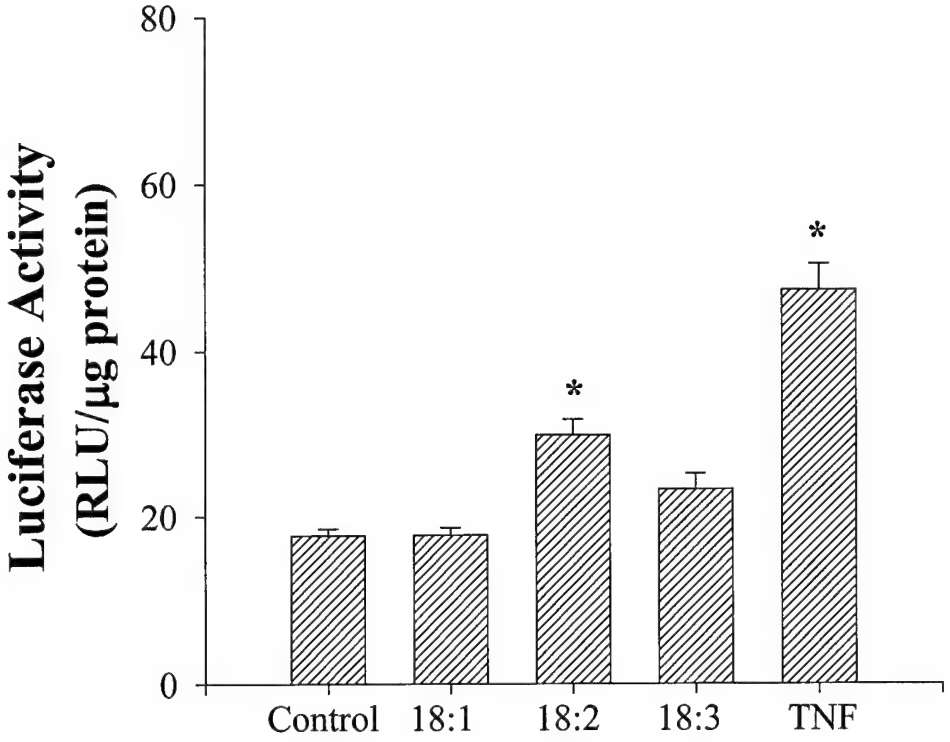
Luciferase Activity
(RLU/μg protein)
80
60
40
20
0
*
*
Control
18:1
18:2
18:3
TNF

Figure 1B

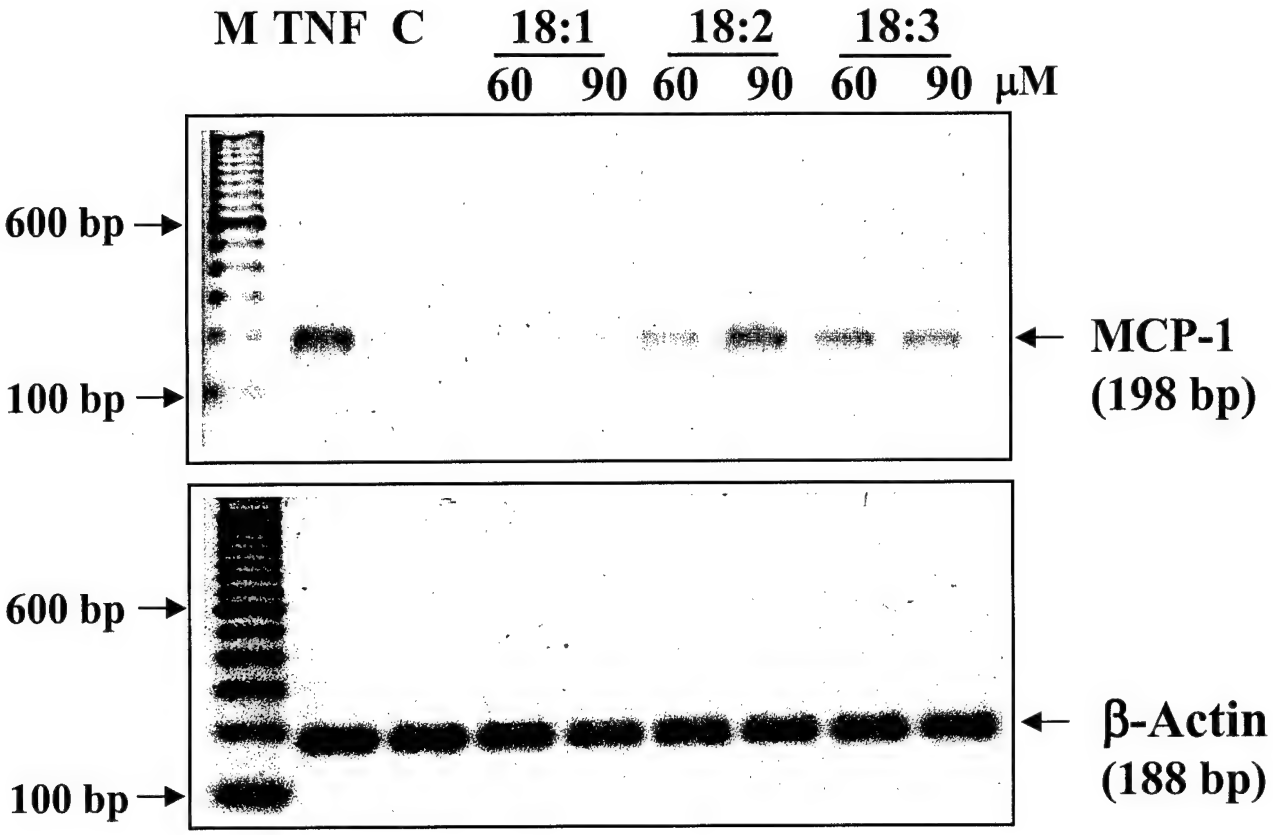
M TNF C
18:1
18:2
18:3
60 90 60 90 60 90 μM
600 bp
100 bp
MCP-1
(198 bp)
600 bp
100 bp
β-Actin
(188 bp)

Figure 2A

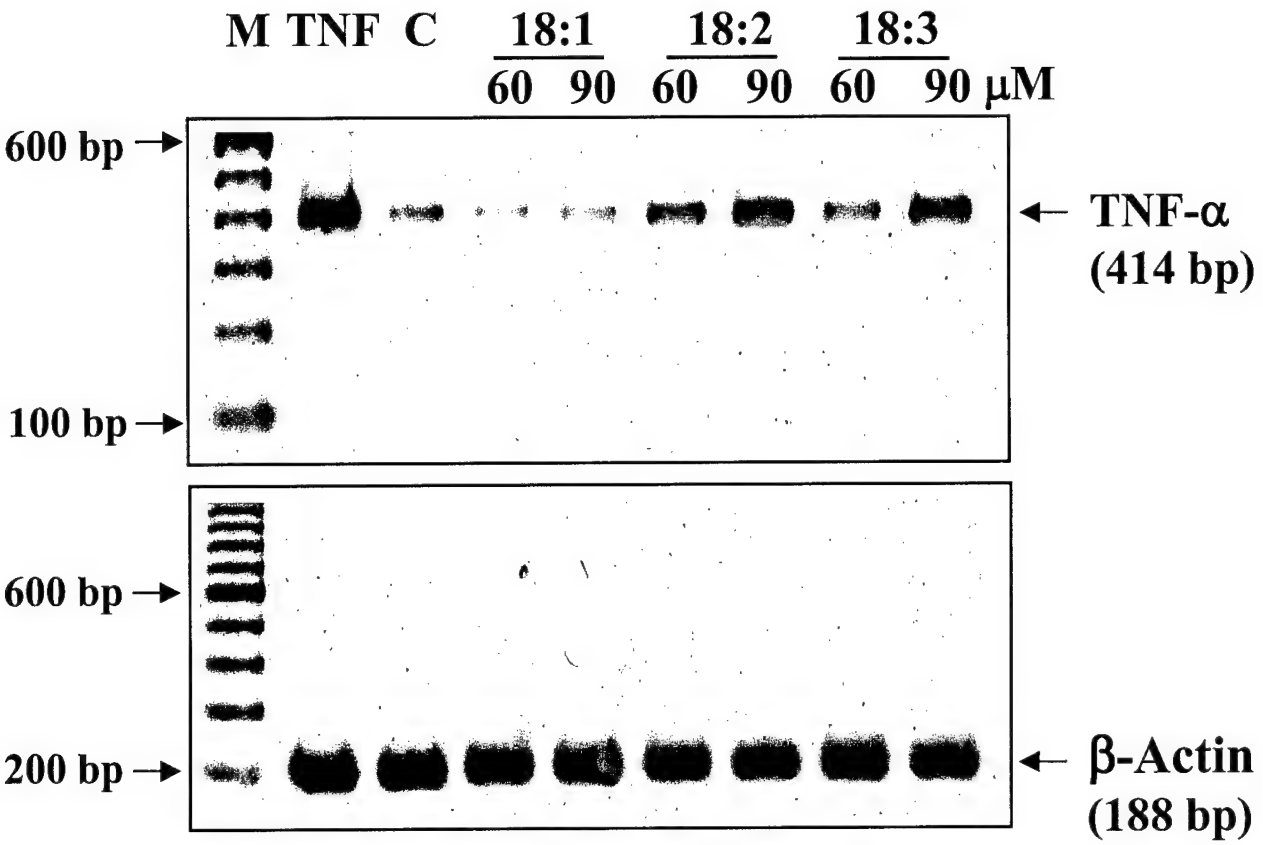
M TNF C
18:1
18:2
18:3
60 90 60 90 60 90 μM
600 bp
100 bp
TNF-α
(414 bp)
600 bp
200 bp
β-Actin
(188 bp)

Figure 2B

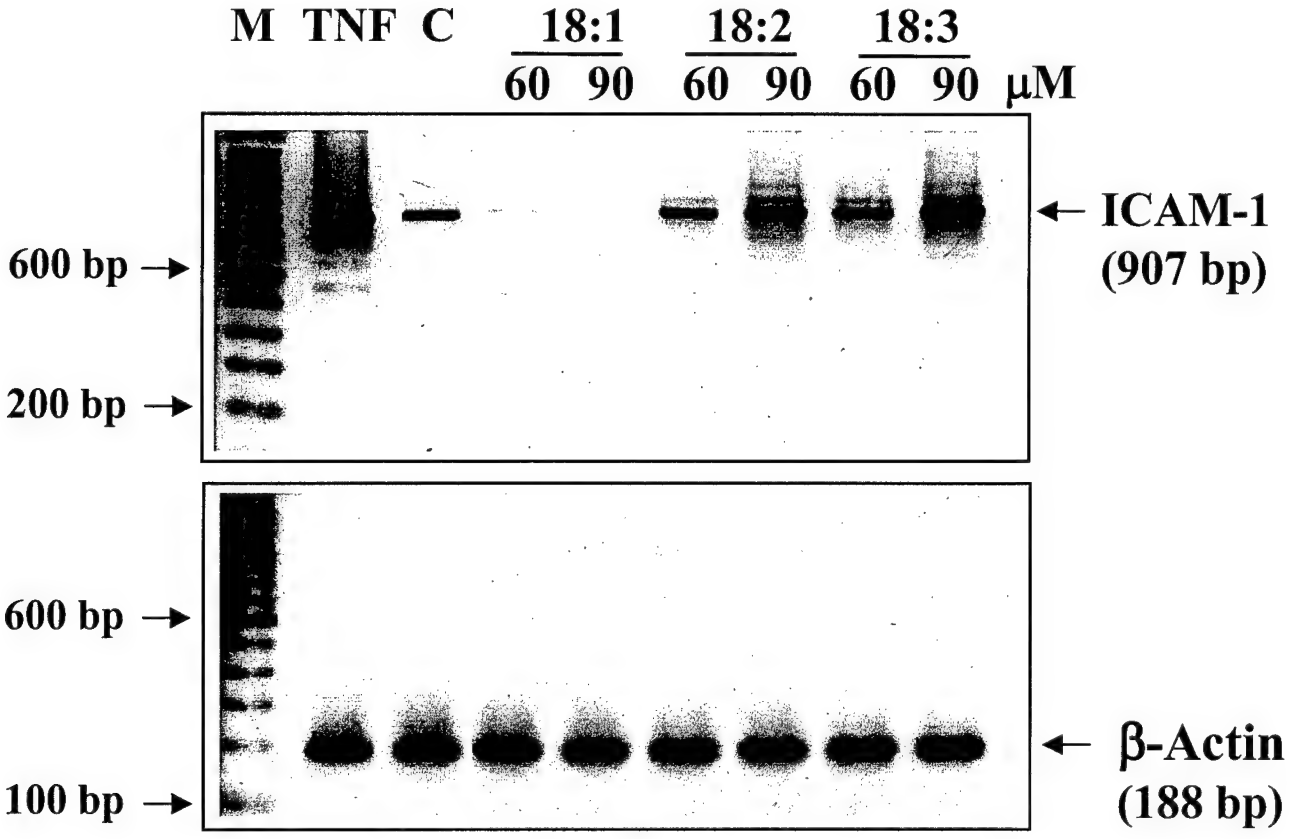
M TNF C
18:1
18:2
18:3
60 90 60 90 60 90 μM
600 bp
200 bp
ICAM-1
(907 bp)
600 bp
100 bp
β-Actin
(188 bp)

Figure 2C

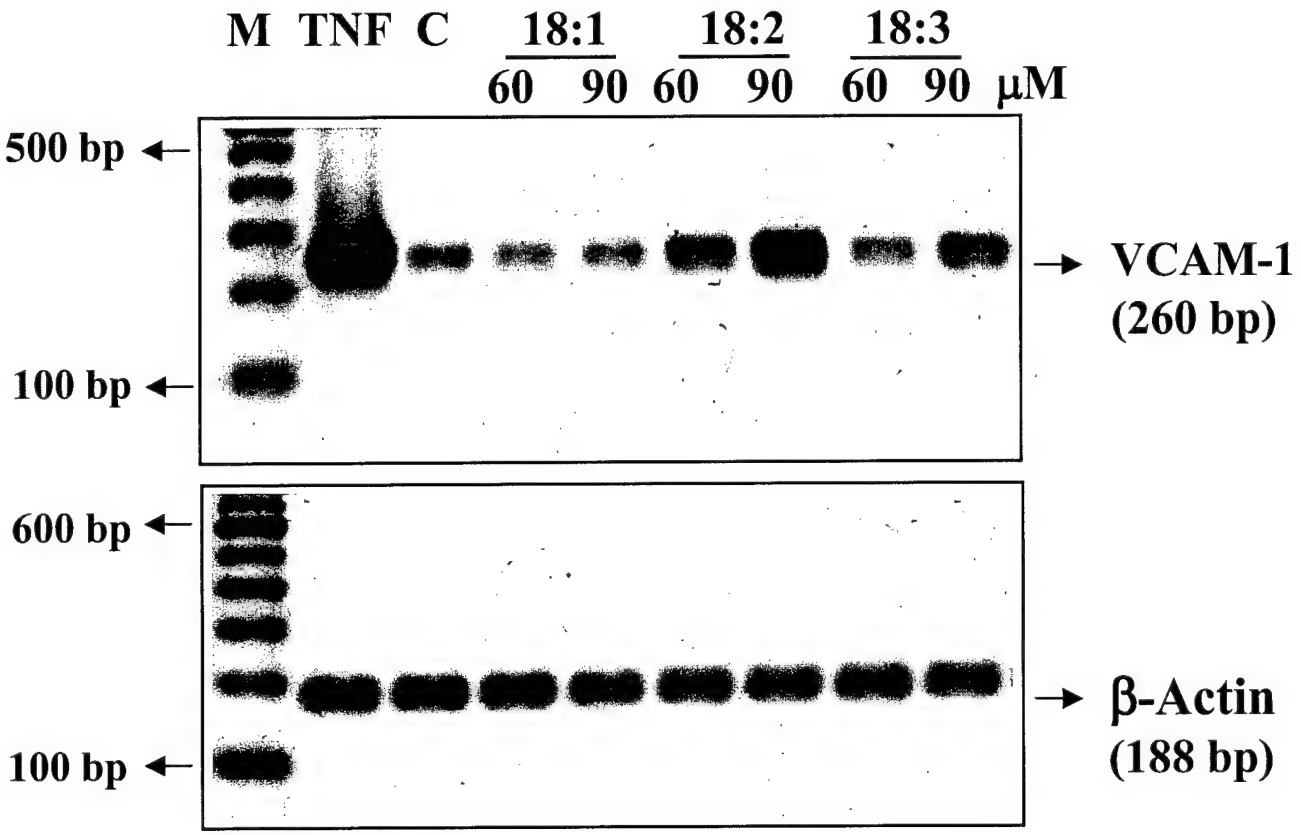
M TNF C 18:1 18:2 18:3
60 90 60 90 60 90 μM
500 bp
100 bp
VCAM-1
(260 bp)
600 bp
100 bp
β-Actin
(188 bp)

Figure 2D

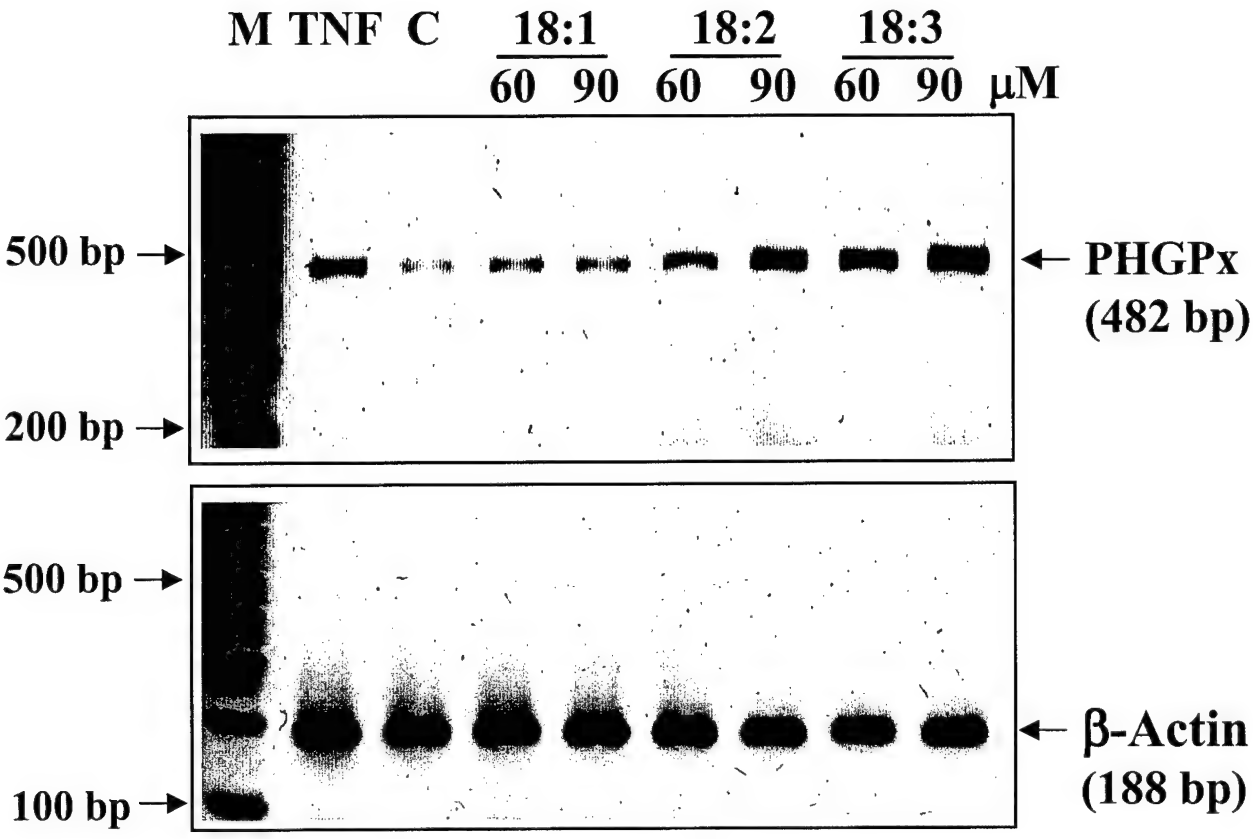
M TNF C
18:1
18:2
18:3
60 90 60 90 60 90 μM
500 bp
200 bp
PHGPx
(482 bp)
500 bp
100 bp
β-Actin
(188 bp)

Figure 3

Measurement of inflammatory properties of fatty acids in human endothelial cells

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Running title: Fatty acids and inflammatory responses

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Introduction

Fatty acids can modulate inflammatory responses in numerous tissues, including the vascular endothelium. At least two different independent pathways can be responsible for these effects. These pathways are linked either to 1) eicosanoid production or 2) redox-regulated gene expression. Traditionally, lipid-mediated cellular inflammatory reactions have been linked to the release of arachidonic acid from the cellular membranes, activation of cyclooxygenases and lipoxygenases with the subsequent overproduction of eicosanoids. These reactions have been relatively well studied and were characterized in detail in several excellent reviews.[1,2,3] Therefore, the following review will focus on dietary fatty acid-induced proinflammatory pathways mediated by the induction of oxidative stress, activation of redox-regulated transcription factors and the inflammatory genes (Figure 1).

Dietary Sources of Fatty Acids

Recent evidence indicates that selected fatty acids can stimulate inflammatory reactions through transcriptional regulation of inflammatory genes, i.e., genes encoding for adhesion molecules and inflammatory cytokines.[4] However, it appears that the effects mediated by individual fatty acids are very specific and influenced by diet and types of dietary fat. It is generally accepted that dietary profiles of fatty acids can significantly influence lipoprotein lipid composition. Thus, diets enriched in specific fatty acids will result in high concentrations of these fatty acids in lipoprotein fractions. Nutritional analyses indicate that the typical Western diet contains 20-25-fold more n-6 (or omega-6) than n-3 (or omega-3) fatty acids. In addition, among n-6 fatty acids, linoleic acid (18:2, n-6) is the major dietary fatty acid present in high concentrations in corn, soy, sunflower, or safflower oils. It is estimated that linoleic acid provides approximately 7-8% of the average dietary energy intake.[1] In addition, linoleic acid is thought to be a predominant substrate for lipid peroxidation processes both in lipoproteins, such as low-density lipoproteins (LDL) as well as in tissues.[5] In contrast to linoleic acid, dietary intake of α-linolenic acid (18:3, n-3), an essential fatty acid of the n-3 family, is relatively low. For example, α-linolenic acid, present in leafy, green vegetables as well as in flaxseed and canola oils, constitutes only 0.3-0.4% of the average dietary energy intake. Oleic acid (18:1, n-9) is another main dietary fatty acid, present in high amounts in olive or sunola oils, as well as in

meat. It is responsible for approximately 8-15% the average dietary energy intake.[1] In contrast to linoleic acid or α-linolenic acid, oleic acid is not an essential fatty acid and can be synthesized from stearic acid by Δ-9 desaturation.[6]

Fatty acids can be hydrolyzed from lipoproteins in a reaction catalyzed by lipoprotein lipase, an enzyme associated with the vascular endothelium. Thus, endothelial cells can be directly exposed to high concentrations of free fatty acids.[7] Evidence indicates that specific free fatty acids can directly affect endothelial cell metabolism and induce potent proinflammatory reactions.[8] Because of their dietary significance and biological potential, our research has concentrated on the effects of 18-carbon fatty acids (such as oleic, linoleic and linolenic acid) on endothelial cell metabolism in relationship to the development of atherosclerosis. Specifically, we study the roles of these fatty acids in the regulation of proinflammatory pathways in human endothelial cells.

Human Endothelial Cell Cultures and Preparation of Fatty Acid-Enriched Media

Background

Human umbilical vein endothelial cells (HUVEC) are the most common primary human endothelial cells available for routine cell culture research. Although these cells are of vein origin, they appear to be well suited for research related to different aspects of vascular biology, including studies on inflammatory responses. For example, HUVEC express all mediators of inflammatory responses, such as genes encoding for adhesion molecules, inflammatory cytokines and chemokines.[9] In addition, HUVEC are susceptible to the development of apoptosis.[10]

Human endothelial cells can also be isolated from other vessels, such as the aorta or the femoral artery. Moreover, a variety of different types of primary endothelial cells are commercially available (e.g., from Clonetics Corp., Walkersville, MD or Cascade Biologics, Portland, OR). Several immortal cell lines of human endothelial cells are also available, such as human microvascular endothelial cells (HMEC-1), which originated from dermal microvascular endothelial cells transfected with the SV-40 large T promoter,[11] or EA.hy926 cell line, produced by fusion of HUVEC with human A549 carcinoma cells.[12]

In most experiments, endothelial cells are exposed to fatty acids at concentrations of 60 or 90 μM, with experimental media albumin concentrations of about 60 μM. Normal plasma

free fatty acid concentrations can range from approximately 90-1200 μM; however, the majority of free fatty acids are bound to plasma components, mostly albumin.[13,14] In fact, the main factor in the availability of fatty acids for cellular uptake is determined by the free fatty acid to albumin ratio. Normally, this ratio can range from 0.15 to 4 in response to various conditions, with an average of approximately 1.[13,14] Thus, the experimental conditions employed in our studies, which result in a free fatty acid to albumin ratio of 1 or 1.5, are within a physiological range.

Solutions for HUVEC Isolation and Culture

Dispase solution: Dispase (2 mg/mL) in M199 enriched with penicillin/streptomycin (400 U/mL) and 3% fetal bovine serum.

Growth medium: M199 with added $NaHCO_3$, pH 7.4 and enriched with heparin, 54.3 U/mL; HEPES, 25 mM; L-glutamine, 2 mM; sodium pyruvate, 1 μM; penicillin, 200 U/mL; streptomycin, 200 μg/mL; amphotericin B, 0.25 μg/mL; endothelial cell growth supplement (ECGS), 0.04 mg/mL; fetal bovine serum, 20 %.

Experimental medium: composition is similar as that of growth medium, except for the serum content. Fetal bovine serum is added to the experimental medium at the final concentration of 10 %.

Hank's balanced salt solution: NaCl, 0.14 M; KCl, 5.36 mM; KH_2PO_4, 0.44 mM; Na_2HPO_4 $7H_2O$, 0.63 mM; $NaHCO_3$, 4.16 mM; D-glucose, 5.55 mM; phenol red sodium salt, 0.001%.

Procedure

Umbilical cords are collected in sterile beakers containing M199 and penicillin and streptomycin at concentrations of 400 U/mL. HUVEC are isolated under aseptic conditions as follows: umbilical cord is placed on sterile gauze and both ends are cleanly cut prior to locating the umbilical vein. Canuli with attached tubings are inserted into the vein from both ends of the cord, and umbilical tape is tightly knotted to uphold the canuli in the vein. Then, blood clots are rinsed from the inside of the umbilical vein by injection of Hank's solution through the canula. After the cleaning of the vein and clamping one end of the umbilical cord, the dispase solution is injected into the vein, allowing the cord to become fully distended. Tubings attached to each canula are clamped and the cords are placed in a sterile beaker

containing Hank's solution and refrigerated overnight for 15 to 18 hours to allow dislodging of endothelial cells. The following day, the dispase-containing cells are collected by rinsing the lumen of the vein with Hank's solution to further dislodge weakly attached cells. The cell suspension is centrifuged for 10 min at 250 x g at room temperature. Then, the pelleted cells are resuspended in growth medium and seeded in a cell culture flask. Two to four hours later, when endothelial cells are fully attached to the surface of the flask, the medium is removed and cells are rinsed gently with Hank's solution to remove any remaining blood cells, and the fresh growth medium is added to the flask. Endothelial cells are cultured at 37 °C in a humid atmosphere of 5% CO_2. Cell cultures are identified as endothelial by their cobblestone morphology and by the uptake of acetylated low-density lipoproteins labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocabocyanine perchlorate (Dil-Ac-LDL). Dil-Ac-LDL bind to the scavenger receptor present on endothelial cells as well as on other cell types, such as macrophages or microglia. However, isolation of endothelial cells from umbilical veins and the subsequent cell culture procedures eliminate a possibility of macrophage or microglia contamination. Thus, under the described conditions, Dil-Ac-LDL uptake combined with morphological appearance of cells can specifically identify endothelial cells. The passage of endothelial cells is performed by washing the cells with Hank's solution and adding trypsin/EDTA at 1:3 split ratio. All our experiments are conducted with cells from passage two.

Measurement of Dil-Ac-LDL Uptake by HUVEC

Dil-Ac-LDL (Molecular Probes, Eugene, OR) is diluted to 10 μg/mL in growth medium and added to cell cultures. Following a 4 h incubation at 37 °C, medium containing Dil-Ac-LDL is removed from the cultures, and cells are washed 3 times with Hank's solution. Then, cells are trypsinized, centrifuged at 500 x g, washed once with PBS and resuspended in PBS to obtain a final concentration of 1 x 10^6 cells/mL. The percentage of fluorescent-labeled cells is measured using an activated cell sorter, FACScan, with the wavelength for excitation and emission set at 514 and 500 nm, respectively. Cells from unlabeled cultures serve as negative controls.

Preparation of Fatty Acid-Enriched Media

Stock solutions of high purity (≥ 99%) fatty acids (Nu-Chek-Prep, Elysian, MN) are prepared in hexane. NaOH (6M, or 30 x molarity of fatty acid) is used for saponification to convert the fatty acids into water soluble form. The desired amount of fatty acid is aliquoted, mixed with 6 M NaOH, and dried under high purity nitrogen gas. The residue is dissolved in 1.0 mL of hot, distilled water, and the solution is immediately transferred to a beaker containing experimental medium. Then, the pH is adjusted to 7.4 with 1.2 M HCl and the medium is sterilized through a syringe-driven filter unit.

Fatty Acid-Induced Oxidative Stress in Endothelial Cells

Background

Among different methods to assess oxidative stress, 2',7'-dichlorofluorescein (DCF) fluorescence appears to be very sensitive and especially useful in experimental settings which include cell cultures.[15] This method allows measurement of cellular oxidation in individual and viable cells directly on cell culture dishes. Cells are loaded with 2',7'-dichlorofluorescin diacetate (DCF-DA), a stable, non-polar compound that readily diffuses into the cells and is converted to a nonfluorescent polar derivative 2',7'-dichlorofluorescin (DCF-H) by intracellular esterases. DCF-H can be oxidized to the highly fluorescent compound DCF by hydrogen peroxide or other peroxides produced by the cells. The intensity of cellular fluorescence can be assessed by a confocal laser-scanning microscope coupled to an inverted microscope.

We demonstrated that DCF fluorescence is a sensitive marker of cellular oxidation induced by fatty acids.[16] Figure 2 shows photomicrographs visualizing the intracellular DCF fluorescence in control endothelial cells as well as in endothelial cells exposed to 90 μM linoleic acid for 6 h. The pseudocolor scale that reflects the levels of the intracellular peroxide tone is arranged in such a way that white color reflects the highest peroxide concentrations, red color high levels, yellow color intermediate, and blue color reflects the lowest levels of intracellular oxidizing compounds. The average pixel intensity is also measured within each field using the "ImageSpace" software supplied by the manufacturer (Molecular Dynamics) and expressed in relative units of DCF fluorescence. DCF fluorescence can be utilized to measure cellular

oxidation in different model systems. In fact, this method has been successfully used in a variety of cell types (e.g., neurons[15]) or treatments (e.g., amyloid β-peptide[17] or interleukin-4[18]).

Although DCF fluorescence is an excellent method to study cellular oxidation, it is variable throughout the cell. Therefore, we perform our measurements constantly one micron below the cell surface. In addition, the loading of the dye may not be uniform across cells cultured in one dish. Thus, it is important to measure DCF fluorescent in a large number of cells in several independent cultures. A standard procedure in our laboratory involves measurements of up to 300 individual cells per culture in 4 independent cultures. In addition, DCF fluorescence is sensitive to pH changes. Therefore, it is important that the pH of each fatty acid-enriched medium is carefully neutralized to normal values before adding such a medium to endothelial cell cultures. Moreover, measurements of DCF fluorescence are performed in the presence of Hank's solution to buffer pH changes which can occur during the procedure.

Procedure

Endothelial cells (3.0×10^5 cells/dish) are plated on polyethylenimine-coated glass-bottom 35-mm dishes (Mat-Tek, Inc., Ashland, MA), cultured for 3-4 days until confluent, and treated with fatty acids. The cells are loaded with 50 μM DCF-DA during the remaining 50 min of the experiment. At the end of the incubation period, cells are washed three times with Hank's solution. Then, 1 mL of Hank's solution is added to cell culture dishes and DCF fluorescence is measured using a confocal laser-scanning microscope (Molecular Dynamics, Sunnyvale, CA) coupled with a Nikon Diaphot inverted microscope (Nikon, Inc., Melville, NY) using 488 nm excitation and 510 nm emission filters. The operating conditions are as follows: objective, 60 x; pinhole aperture, 50 μm; image size 1024 x 1024 pixels; pixel size 0.21 μm. Average pixel intensity is measured within each individual cell and expressed in the relative units of DCF fluorescence. Values are expressed as mean ± SEM of individual cells from 3 or 4 separate plates.

Other Methods to Assess Cellular Oxidation Status in Fatty Acid-Treated Endothelial Cells

Popular methods to assess cellular oxidative stress include measurements of thiobarbituric acid-reactive substances (TBARS), lipid hydroperoxides, conjugated dienes, and

4-hydroxynonenal (HNE).[19,20] Among them, measurement of TBARS is still used most frequently. The principle of the method is based on the reaction between malondialdehyde (MDA), an aldehyde product of lipid peroxidation, with thiobarbituric acid (TBA) in high temperature, typically 100 °C. This method is criticized as a marker of lipid peroxidation, because normally MDA is only a minor product of lipid peroxidation and the majority of detectable MDA is formed from hydroperoxides during heating in the reaction with TBA. In addition, MDA is formed from fatty acids which contain a minimum of three double bonds. Thus, MDA is not generated from linoleic acid, which appears to be the main fatty acid involved in lipid peroxidation. Finally, several compounds can react with TBA in addition to MDA. A partial list of these compounds includes sialic acid, prostaglandins, thromboxanes, deoxyribose and other carbohydrates.[19,20]

Lipid hydroperoxides are formed as intermediates of lipid peroxidation. A popular method to assess lipid hydroperoxide level is based on peroxide-mediated oxidation of ferrous (Fe^{2+}) to ferric (Fe^{3+}) iron. Ferric iron can bind to xylenol orange to produce a chromophore that can be quantitated at 560 nm (FOX assay).[21] We successfully used this method in our studies on linoleic acid-induced oxidation of cultured endothelial cells.[22]

Lipid peroxidation may also be simply assessed by accumulation of the conjugated dienes. This method is based on the principle that during initiation of lipid peroxidation, isolated double bonds in fatty acid molecules are shifted to conjugated double bonds, which are detectable at 234 nm. Because of the simplicity of detection, measurements of conjugated dienes are useful for the continuous monitoring of lipid peroxidation to detect the susceptibility of biological samples to oxidation. This approach requires stimulation of cellular oxidation, e.g., by adding copper or iron ions.[23]

HNE is another aldehyde product of lipid peroxidation.[20] In contrast to MDA, it can be generated during peroxidation of linoleic acid. In fact, we indicated that HNE is formed in HUVEC exposed to this fatty acid for 24 h. However, it should be noted that arachidonic acid, which is more unsaturated than linoleic acid, appears to be a better substrate for HNE. Levels of HNE in endothelial cells can be semi-quantitatively determined by immunocytochemistry or Western blot and quantitatively by HPLC.[24]

In general, FOX method, TBARS levels, formation of conjugated dienes and, to a lesser extent, production of HNE are much less sensitive than DCF fluorescence and can be performed

only on large numbers of endothelial cells. In addition, they require extensive manipulations with cells, such as harvesting or sonication, which are inevitably connected with creation of artificial oxidation in a test tube. Therefore, from our experience, DCF fluorescence appears to be the method of choice to study fatty acid-induced cellular oxidation in cultured endothelial cells.

Fatty Acid-Induced Alterations of Glutathione Levels and Cellular Redox Status

Background

Increased cellular oxidation results in alteration of the cellular redox status, which can be detected by the ratio of oxidative to reduced glutathione (GSSG and GSH, respectively). It is well known that oxidative stress results in decreased levels of total glutathione and increased concentrations of GSSG. Thus, the ratio of GSSG/GSH is recognized as a sensitive marker of cellular oxidative stress. In addition, because glutathione is the major nonprotein sulfhydryl compound, it plays a critical role in the maintenance of the cellular redox status.[25] In fact, the equilibrium between GSSG and GSH can regulate activation of redox-regulated transcription factors, such as nuclear factor-κB (NF-κB) or activator protein-1 (AP-1). Our research indicated that treatment of endothelial cells with specific fatty acids can lead to decreased levels of total glutathione as well as alterations of GSSG/GSH ratio. We also found a direct correlation between changes in cellular glutathione levels in fatty acid-treated endothelial cells and other methods of assessing cellular oxidative status which are described above.[26]

Among several methods to determine cellular glutathione content, an enzymatic recycling assay first described by Tietze appears to be simple and reliable.[27] There are several versions of this method, with a recent modification which allows to perform the measurements using a microtiter plate reader.[28] Specificity of this method for glutathione assessment is ensured by highly specific glutathione reductase, which is added to the reaction mixture. This method also can be adapted to assay for GSSG by prior derivatization of GSH by adding 4-vinylpyridine. Then, levels of GSH can be calculated by a simple subtraction of GSSG concentration from total glutathione content. The detailed procedure to measure total cellular glutathione using a plate reader is given below.[28]

Another popular method to detect cellular glutathione level is based on the reaction of GSH and/or GSSG with *ortho*-phthalaldehyde (OPT) followed by fluorescence detection. The original method describing this approach was criticized because it markedly overestimated levels of GSSG.[29] However, a recently developed modification, in which GSSG is separated by HPLC and where the reaction with OPT is performed at high pH, appears to avoid these limitations.[30]

Procedure

Endothelial cells are cultured until confluence and treated with fatty acids at the concentration of 60-90 μM for up to 24 h. At the end of the incubation period, cells are washed with PBS and scraped into 2.25% 5-sulfosalicylic acid. After centrifugation at 14,000 × g for 20 min at 4 °C, the supernatant is used for the determination of total glutathione, whereas the pellet is dissolved in 0.2 M NaOH containing 0.1% SDS for protein concentration analysis. Levels of total glutathione in acid-soluble fractions are determined by enzymatic recycling assay in the presence of 0.15 mM 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), 0.2 mM NADPH, and 1.0 unit of glutathione reductase/mL of assay mixture in 30 mM sodium phosphate buffer (pH 7.5), containing 0.3 mM EDTA. Total glutathione is estimated by monitoring the rate of formation of the chromophoric product 2-nitro-5-thiobenzoic acid at 405 nm. The glutathione content in samples is calculated on the basis of the standard curve obtained with known amounts of glutathione and expressed in nmol of glutathione per mg of cellular protein.

Fatty Acid-Induced Activation of Redox-Regulated Transcription Factors in Human Endothelial Cells

Background

Induction of cellular oxidative stress and/or changes of intracellular glutathione levels can trigger signal transduction pathways via activation of redox-responsive transcription factors and, hence, the transcription of specific genes. Among oxidative stress-responsive transcription factors, NF-κB and AP-1 appear to be most important. Indeed, increased activities of AP-1 and NF-κB are considered to be a part of a general regulation of gene expression by oxidative stress. NF-κB is composed of homo- or heterodimeric complexes of at least five distinct subunits, such as p50, p52, p65 (RelA), c-Rel, and Rel-B; however, the p50/p65 heterodimer is the predominant

form of this transcription factor.[31] NF-κB binding sites were identified in the promoter regions of genes encoding for adhesion molecules (intracellular adhesion molecule-1, ICAM-1; vascular cell adhesion molecule-1, VCAM-1; or E-selectin) and inflammatory cytokines (such as tumor necrosis factor-α, TNF-α; IL-1β, IL-6, or IL-8), growth factors and chemokines. Although other transcription factors are also required for expression of these genes, NF-κB constitutes an important component of their transcriptional regulation. It is interesting that the expression of inflammatory cytokines is dependent on activated NF-κB and, in turn, these cytokines can stimulate activation of this transcription factor. Thus, it appears that inflammatory cytokines use NF-κB to amplify their own signals.[32]

Activation of AP-1 also can be implicated in the induction of the inflammatory genes. AP-1 is a family of basic domain/leucine zipper transcription factors that have been characterized for the specific binding to and transactivation through a cis-acting 12-O-tetradecanoyl phorbol-13-acetate (TPA) response element. AP-1 is composed of the Jun and Fos gene products, which can form heterodimers (Jun/Fos) or homodimers (Jun/Jun). It was shown that c-Fos/c-Jun binding activity towards AP-1 sites is regulated by the oxidative status of cysteine residues of c-Fos and c-Jun proteins (Fos Cys-154 and Jun Cys-272, respectively). Oxidation of cysteine residues can convert c-Fos and/or c-Jun into inactive forms. In contrast, a reduction of these residues can re-activate c-Fos/c-Jun binding activity.[33] AP-1 binding sites were identified in the promoter regions of genes encoding for inflammatory cytokines such as IL-6,[34] and adhesion molecules such as ICAM-1[35], VCAM-1,[36] and E-selectin.

Evidence indicates that not only NF-κB and AP-1 but also other transcription factors may belong to the family of the transcription factors whose activity is regulated by the cellular redox status. For example, our studies indicate that SP-1 and STAT1α may be regulated by cellular oxidative status.[18,37]

Electrophoretic mobility shift assay (EMSA) is utilized to determine the binding interaction of transcription factors with their specific DNA sequences. This assay is a simple, relatively rapid, and very sensitive method to perform. EMSA is based on the principle that specific protein-DNA binding complexes have higher molecular weight than unbound oligonucleotide probes and migrate slower during a nondenaturing polyacrylamide gel electrophoresis (PAGE). The binding specificity of the bands corresponding to the specific transcription factors is established using at least three different experimental approaches: i)

competition binding with the molar excess of unlabeled oligonucleotide probes, ii) binding with mutant oligonucleotides, and iii) supershift with antibodies against specific subunits of individual transcription factors.

Using EMSA, we demonstrated that treatment of cultured endothelial cells with specific free fatty acids resulted in an increase in NF-κB or AP-1 binding activity as well as transactivation of these transcription factors. Figure 3A depicts the effects of linoleic acid on the binding activity of NF-κB in human endothelial cells. A slight endogenous activity of NF-κB is observed in untreated control cell cultures (lane 2). However, when cells are stimulated with linoleic acid or lipopolysaccharide (LPS; positive control), a significant increase of binding activity is detected (lanes 3-6). The DNA binding is specifically inhibited by an unlabeled competitor DNA containing the consensus NF-κB sequence (lane 7). Identities of the bands also can be confirmed by antibodies against specific NF-κB subunits, i.e., anti-p50 and anti-p65 (Figure 3B, lane 3-4).

Reagents, Buffers, and Equipment Required for EMSA

Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4

Lysis buffer: 10 mM Tris-HCl, pH 8.0, 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 100 μM phenylmethylsulfonyl fluoride (PMSF), 0.1% NP-40

Nuclear extract buffer: 20 mM Tris-HCl, pH 8.0, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol

Redivue adenosine 5'-[γ-^{32}P]triphosphate, triethylammonium salt, 10 mCi/mL (Amersham Pharmacia Biotech, Piscataway, NJ)

0.25 × TBE buffer: 50 mM Tris-Cl, 45 mM boric acid, 0.5 mM EDTA, pH 8.4

V16 Vertical gel electrophoresis apparatus, 0.8-mm-thick gel (Life Technologies, Gaithersburg, MD) or similar instrument

X-OMAT AR Kodak autoradiography film (Kodak, Rochester, NY)

Procedure

A. Isolation of Nuclear Extracts (All steps are performed on ice unless otherwise specified)[38]

1. Endothelial cell cultures (at least 6.0×10^6 cells per group) are treated with fatty acids for 0.5 - 6 h, trypsinized, and collected by centrifugation at 2,500 rpm for 4 min at 4 °C. The pellet is washed once with PBS.
2. The cells are lysed in 1 mL of Lysis buffer for 5 min on ice and centrifuged at 2,500 rpm for 4 min at 4 °C to collect nuclei. Then, the nuclear pellets are washed with 1 mL of Lysis buffer without NP-40.
3. The nuclear pellets are lysed in 100 μL of Nuclear extract buffer for 10 min on ice and centrifuged at 14,000 rpm for 15 min at 4 °C.
4. Supernatants which contain nuclear extracts are frozen immediately in liquid nitrogen. Then, they can be transferred to -80 °C freezer and stored for 2 weeks.

B. 5'-End Labeling of Oligonucleotides with [γ-^{32}P]-ATP

1. Double-stranded oligonucleotide probes are labeled with [γ-^{32}P]-ATP using bacteriophage T4 polynucleotide kinase. The reaction mixture consists of 70 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 5 pmoles of double-stranded oligonucleotides, 30 μCi of [γ-^{32}P]-ATP, and 20 units of T4 polynucleotide kinase (Promega, Madison, WI) in a total volume of 20 μL. The reaction mixture is incubated for 1 h at 37 °C
2. Following incubation, T4 polynucleotide kinase is inactivated by placing the tube for 10 min at 68 °C on a heat block.
3. Unincorporated nucleotides are removed by gel filtration chromatography using mini Quick Spin Oligo Columns (Boehringer Manheim Corporation, Indianapolis, IN).

C. Binding Reaction and Electrophoresis

1. Binding reactions are performed in a 20 μL volume containing 4 - 10 μg of nuclear protein extracts, 10 mM Tris-Cl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.1 mM dithiothreitol, 10% glycerol, and 2 μg of poly[dI-dC], which is used as a nonspecific competitor. After adding the reagents, the mixture is incubated for 25 min at room temperature.
2. Then, 40,000 cpm of ^{32}P-labeled specific oligonucleotide probe is added, and the binding mixture is incubated for 25 min at room temperature. Competition studies and supershift experiments are performed by the addition of a molar excess of unlabeled oligonucleotide probes or antibodies against specific transcription factors to the binding reaction.

3. Resultant protein-DNA complexes are electrophoresed on a non-denaturing 5% polyacrylamide gel (pre-run for 2 h at 150 V) using 0.25 × TBE buffer for 2 h at 150 V.
4. The gel is transferred to Whatman® 3MM paper, dried on a gel dryer, and exposed to a X-ray film overnight at -70 °C with an intensifying screen.

Fatty Acid-Induced Inflammatory Genes in Endothelial Cells

Background

Fatty acid-induced inflammatory reactions in endothelial cells are mediated by production of chemokines (e.g., monocyte chemoattractant protein-1; MCP-1), inflammatory cytokines (e.g., TNF-α), and adhesion molecules (e.g., ICAM-1 or VCAM-1)[39]. Expression of these inflammatory mediators and their effects are closely interrelated. For example, ICAM-1 and VCAM-1 facilitate leukocyte adhesion to the vascular endothelium and both MCP-1 and, to a lesser extent, TNF-α are potent chemoattractive factors, which play a significant role in recruiting lymphocytes and monocytes into the vessel wall.[40,41] In addition, TNF-α is a strong inducer of inflammatory reactions and can stimulate overexpression of MCP-1, inflammatory cytokines, as well as ICAM-1 and VCAM-1.[42] Recently, we obtained evidence that selective dietary fatty acids can induce expression of the inflammatory genes, such as ICAM-1, VCAM-1, MCP-1 or TNF-α in endothelial cells.[39] RT-PCR with specific primer pairs (Table 1) is a very suitable experimental technique to perform these analyses.

Figure 4 depicts RT-PCR analysis of the effects of treatment with selected unsaturated fatty acids for 3 h on MCP-1 gene expression in HUVEC. Among tested fatty acids, linoleic acid stimulated the most pronounced overexpression of the MCP-1 gene. Indeed, expression of this gene in endothelial cells treated with 90 μM linoleic acid was in the range of that observed in cells exposed to 20 ng/mL of TNF-α, which was used as a positive control. Expression of the MCP-1 gene was also increased in endothelial cells treated with linolenic acid. In contrast, expression of this gene in endothelial cells exposed to oleic acid appeared to be within or even below the control range observed in non-stimulated endothelial cells. Fatty acid-mediated alterations of other inflammatory genes result in the same or similar pattern of changes.

The RT-PCR technique can be divided into three steps; A) isolation of total RNA, B) reverse transcription, and C) polymerase chain reaction. For the isolation of high purity total

RNA from cell cultures, TRI REAGENT™ is utilized. TRI REAGENT™ is a mixture of guanidine thiocyanate and phenol in a monophase solution, which effectively dissolves DNA, RNA, and protein in cell lysates. Then, isolated total RNA is utilized for the reverse transcription (RT) and polymerase chain reaction (PCR) amplification of a specific target RNA. AMV reverse transcriptase, RNA-dependent DNA polymerase from Avian myeloblastosis virus, is utilized to synthesize the first single-stranded cDNA from isolated RNA. In addition, *Taq* DNA polymerase, a thermostable DNA-dependent DNA polymerase from *Thermus aquaticus*, is used to synthesize the second strand cDNA and for DNA amplification.

Reagents and Equipment

TRI REAGENT™ (Sigma, Saint Louis, MO)

Nuclease-Free Water (Promega, Madison, WI)

Reverse Transcription System (Promega, Madison, WI)

Taq PCR Master Mix Kit (Qiagen, Valencia, CA)

GeneAmp® PCR System 9700 (The Perkin-Elmer Corporation, Norwalk, CT) or similar instrument

Agarose, Ultra Pure (Life Technologies, Gaithersburg, MD)

SYBR® Green I (Molecular Probes, Inc., Eugene, OR)

Horizontal Gel Electrophoresis System (e.g., Horizon 11·14, Life Technologies, Gaithersburg, MD) or similar instrument

Phosphorimager (Fuji FLA-2000, Stamford, CN) or similar system

Procedure

A. Isolation of Total RNA for RT-PCR

1. Lysis of cultured monolayer cells

Endothelial cell cultures (3.0×10^6 cells per 100-mm culture dish) are treated with fatty acids, washed with PBS and lysed by the direct addition of 1 mL of TRI REAGENT to culture dishes. The cell lysate is passed several times through a pipette to form a homogenous lysate. The homogenate is centrifuged at 12,000 × g for 10 min at 4 °C to remove the insoluble material such as extracellular membranes, polysaccharides, and

high molecular weight DNA. After centrifugation, the clear supernatant is transferred to a fresh tube and allowed to stand for 5 min at room temperature.

2. Phase separation

0.2 mL of chloroform is added to the cell lysate, shaken vigorously for 15 sec, and allowed to stand for 10 min at room temperature. Then, the resulting mixture is centrifuged at 12,000 × g for 15 min at 4 °C. Centrifugation separates the mixture into the following 3 phases: a red lower organic phase (protein part), a white interphase (DNA part), and a colorless upper aqueous phase (RNA part). The upper phase is transferred to a fresh tube. This is done very carefully in order not to disrupt the interphase or the lower phase.

3. Isolation of total RNA

0.5 mL of isopropanol is added to the tube containing the transferred upper aqueous phase (RNA part), mixed gently by inverting several times, and allowed to stand for 10 min at room temperature. The mixture is centrifuged at 12,000 × g for 10 min at 4 °C to precipitate RNA to the side and bottom of the tube. The supernatant is discarded and the white RNA pellet is washed with 1 mL of 75% ethanol. The mixture is briefly vortexed and centrifuged at 7,500 × g for 5 min at 4 °C. Then, the supernatant is very carefully discarded and the RNA pellet is air-dried for 10 min. Approximately 20 - 40 µL of nuclease-free water is added to the RNA pellet and mixed by repeated pipetting at 55 °C for 10 min to facilitate dissolution. Finally, the sample is incubated for 5 min at 70 °C and quickly cooled on ice. This procedure prevents RNA from possibly forming secondary structures, which may interfere with the reverse transcription. Concentration and purity of total RNA is determined spectrophotometrically by measuring the absorbance at 260 nm (one absorbance unit at 260 nm equals 40 µg RNA/mL) and 280 nm. The ratio between the absorbance at 260 and 280 nm reflects RNA purity and should be ≥ 1.7. The sample concentration of RNA is adjusted to the final level of 1 µg RNA/µL.

B. Reverse Transcription (RT)

One microgram of RNA, isolated from endothelial cells as described above, is reverse-transcribed at 42 °C for 60 min, followed by a 5 min incubation at 99 °C and immediately

cooled on ice. The complete reaction mixture for reverse transcription consists of 1 μg of isolated total RNA, 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1 mM dNTP, 1 unit/μL of recombinant RNasin ribonuclease inhibitor, 15 units/μg of AMV reverse transcriptase, and 0.5 μg of oligo$(dT)_{15}$ primer. The total volume of this mixture is 20 μL, adjusted with distilled water.

C. Polymerase Chain Reaction (PCR) and Agarose Gel Electrophoresis

To perform PCR amplification, 2 μL of the reverse transcriptase reaction is mixed with a Taq PCR Master Mix Kit (Qiagen, Valencia, CA) and 20 pmol of primer pairs in a total volume of 50 μL, adjusted with distilled water. Table 1 depicts sequences of the primer pairs that are used for PCR amplification of most common human inflammatory genes in endothelial cells. Expression of β-actin is determined as a housekeeping gene. Expression of the studied genes is determined in the linear range of PCR amplification, specific for an individual PCR reaction. PCR products are separated by 2% agarose gel electrophoresis, stained with SYBR® Green I (Molecular Probes, Eugene, OR) and visualized using phosphoimaging technology (FLA-2000, Fuji, Stamford, CT). For quantitation, intensity of the band corresponding to the specific inflammatory gene is related to the intensity of the band which reflects expression of the β-actin mRNA.

Fatty Acid-Induced Protein Expression of Inflammatory Mediators in Endothelial Cells

Protein expression of the inflammatory mediators is determined using either flow cytometry or ELISA. Flow cytometry is employed to assay expression of adhesion molecules, such as ICAM-1 or VCAM-1 which are present on the endothelial cell surface. FITC-labeled specific monoclonal antibodies (e.g., from R&D, Minneapolis, MN or BD PharMingen, San Diego, CA) appear to be most suitable for this method. A protocol to determine expression of adhesion molecules in fatty acid-treated endothelial cells by flow cytometry is given below.

ELISA kits, commercially available from several companies (e.g., R&D, Minneapolis, MN or Amersham Pharmacia Biotech, Piscataway NJ), allow for quantitative determination of soluble inflammatory mediators, such as chemokines (MCP-1) or inflammatory cytokines (e.g., TNF-α, IL-1β, IL-6 or IL-8) in culture media. Adhesion molecules shed from the surface of

endothelial cells also can be present in cell culture media and can be assayed by commercially available ELISA kits. However, determinations of soluble ICAM-1 or soluble VCAM-1 are more frequent in clinical studies, where serum levels of these molecules can serve as a marker of adhesion molecule expression.

Flow Cytometry Procedure to Detect Expression of Adhesion Molecules

Endothelial cells are cultured on six-well plates, grown to confluence, and treated with fatty acids for 12 h or 24 h. Cells are washed with Hank's solution and gently harvested by trypsin/EDTA. It is important to note that excess and repeated exposure to trypsin can damage the endothelial cell surface and interfere with the results. Then, endothelial cells are washed twice with ice-cold PBS and incubated for 1 h on ice with saturating amounts of specific monoclonal anti-human antibody labeled with FITC. FITC-labeled anti-human IgG is used as the isotype control. After incubation with antibodies, samples are washed twice with ice-cold PBS, suspended in 200 μL PBS and analyzed with 10,000 cells per sample in a fluorescence-activated cell sorter (Becton Dickinson, San Jose, CA). Following correction for unspecific binding (isotype control), intensity of fluorescence or percentage of positively stained cells can be utilized as the indicator of adhesion molecule protein expression.

Transient Transfection of Endothelial Cells and Dual Luciferase Reporter Gene Assay

Transfections (physical or chemical methods of introducing foreign DNA into eukaryotic cells) and reporter gene assays provide powerful experimental tools to study mechanisms of gene regulation. During transient transfections, plasmid DNA is introduced into a cell population, and expression of the reporter gene is studied shortly after the transfection procedure, usually within 24-72 hours. Transfection methods include calcium-phosphate precipitation, electroporation, detergent-DNA complexes, DNA-DEAE complexes, microinjection, virus-mediated transfection, introduction of DNA via particle bombardment and lipid-mediated transfection. In transfections performed *in vitro* in cultured cells, cationic lipids have become standard carriers of plasmid DNA. This method takes advantage of the associations of negatively charged DNA with positively charged liposomes to form a lipid-DNA complex, which can be introduced into cells relatively easily.

Endothelial cells, in general, are difficult to transfect. This may be related to the fact that these cells represent a physiologic barrier against invasion of the vessels and underlying tissues by exogenous substances. However, under carefully controlled experimental conditions, liposome-mediated transfection can be suitable to study transactivation of redox-regulated transcription factors and mechanisms of expression of the inflammatory genes in fatty acid-treated human endothelial cells. Recently, we optimized transfection conditions in human endothelial cells to achieve high-efficiency transient transfections.[43]

Firefly luciferase has been recognized to be the reporter gene of choice for transfection studies in cells resistant to uptake of foreign DNA, such as endothelial cells.[44] The transgene is simple to measure and has no background levels in human tissues. Determination of luciferase activity also has the advantage of being several orders of magnitude more sensitive than other common reporter gene assays, such as activities of chloramphenicol acetyltransferase, β-galactosidase or alkaline phosphatase. To correct for variations in transfection efficiency, a co-transfection with an internal control plasmid should be performed. β-galactosidase or *Renilla* luciferase expression vectors are examples of plasmids which can be used as internal controls. When cells are transfected with constructs encoding for firefly luciferase as a targeted reporter gene and co-transfected with the *Renilla* luciferase expression vector, both a dual luciferase reporter assay system and a luminometer with double injector are required to perform transgene measurements. This system takes advantage of the fact that firefly luciferase and *Renilla* luciferase have distinct enzyme structures and substrate specificity. Thus, their activities can be sequentially measured in the same sample.

Reagents and Equipment

Renilla luciferase expression plasmid used as internal control (pRL-SV40 control vector, Promega, Madison, WI)

Dual-Luciferase® Reporter Assay System (Promega, Madison, WI)

Luminometer fitted with two reagent injector (TD-20/20, Turner Designs, Sunnyvale, CA or similar model)

Procedure

A. Endothelial Cell Cultures

Endothelial cells are seeded on 6-well plates (1.0 - 2.0 × 10^5 cells per well) and cultured for 24-48 h until the cultures reach ~60 % confluence.

B. Preparation of the transfection solution (1 mL per each well)

1 Specific firefly luciferase reporter plasmid (5-10 μg/mL) and *Renilla* luciferase expression plasmid (0.25-0.5 μg/mL, pRL-SV40 control vector) are mixed thoroughly with serum-free medium in a sterile tube in a volume of 0.5 mL/well.

2 In a separate sterile tube, a cationic liposome, such as pFx-7 (36 μg/mL), or DMRIE-C (40 μg/mL) is mixed thoroughly with serum-free medium in a volume of 0.5 mL/well.

3. The plasmid and liposome solutions (prepared in steps 1 and 2, respectively) are combined in a single tube, mixed thoroughly, and incubated for 30 min at 37 °C to allow the formation of liposome/DNA complexes.

C. Transient transfection procedure

1. Growth medium is removed from endothelial cell cultures and the monolayers are washed three times with serum-free medium.

2 The transfection solution (prepared as described above) is added to each well in a volume of 1 mL/well. Plates are returned to the cell culture incubator, and endothelial cells are transfected for 1.5 h.

3 The transfection solution is carefully removed from each well, the cells are gently overlaid with 2 mL of normal growth medium, and returned to the incubator for 24 h at 37 °C.

D. Treatment of transfected cells with fatty acids and determination of dual luciferase activity

1. Cultures are washed with Hank's solution, and 2 mL of experimental media enriched with fatty acids are added into each well of the 6-well plate. Cells are treated with fatty acids for 16 - 24 h.

2. After incubation time, the cells are washed twice with PBS and lysed in 500 μL Passive Lysis Buffer while shaking the plates for 15 min at room temperature.

3. The cell lysates are centrifuged at 12,000 rpm for 1 min to remove cell debris, and cell lysates are transferred to fresh tubes.

4. The firefly and *Renilla* luciferase activities are determined in 10 - 20 μL of cell lysates using a luminometer with dual injector system. Injector #1 is set up to deliver 100 μL/tube of Luciferase Assay Reagent II for determination of the firefly luciferase

activity, and Injector #2 is set up to deliver 100 μL/tube of Stop & Glo Reagent for determination of the *Renilla* luciferase activity.

5. Relative luciferase activity is calculated as the ratio of firefly luciferase activity to *Renilla* luciferase activity.

Conclusions

Using the described methods, we determined that treatment of human endothelial cells with selected dietary fatty acids can induce oxidative stress, decrease cellular glutathione content, activate redox-responsive transcription factors, and induce expression of the inflammatory mediators, such as MCP-1, inflammatory cytokines (IL-6, IL-8, and TNF-α) and adhesion molecules (ICAM-1 and VCAM-1). However, the effects exerted by dietary fatty acids were highly specific. Among studied fatty acids, treatment with linoleic acid induced the most significant oxidative stress, alterations of cellular redox status and induction of the inflammatory genes.[9,24,38] The proinflammatory effects of linolenic acid were less pronounced. In contrast, exposure of human endothelial cells to oleic acid diminished expression of the inflammatory genes. These results demonstrate that specific unsaturated dietary fatty acids, such as linoleic acid, the parent omega-6 fatty acid which also is a major fatty acid in common vegetable oils, can stimulate inflammatory responses in vascular endothelial cells. These proinflammatory effects of selected fatty acids illustrate the significance of dietary lipids in the development, progression or prevention of chronic vascular diseases, such as atherosclerosis.

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Table 1. Sequences of the primer pairs employed in the RT-PCR reactions

Studied inflammatory gene	**Sequences of the primer pairs (5' - 3')**
MCP-1*	Forward: CAG CCA GAT GCA ATC AAT GC Reverse: GTG GTC CAT GGA ATC CTG AA
TNF-α*	Forward: AGC CTC TTC TCC TTC CTG AT Reverse: AGT AGA TGA GGG TCC AGG AG
ICAM-1	Forward: GGT GAC GCT GAA TGG GGT TCC Reverse: GTC CTC ATG GTG GGG CTA TGT CTC
VCAM-1[1]	Forward: ATG ACA TGC TTG AGC CAG G Reverse: GTG TCT CCT TCT TTG ACA CT
β-Actin (a housekeeping gene)[2]	Forward: AGC ACA ATG AAG ATC AAG AT Reverse: TGT AAC GCA ACT AAG TCA TA

Abbreviations: ICAM-1, intracellular adhesion molecule-1; MCP-1, monocyte chemoattractant protein-1; TNF-α, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1.

*Primer pairs purchased from R&D Systems (Minneapolis, MN)

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Figure legends

Figure 1. Schematic diagram of the proinflammatory pathways induced by dietary fatty acids in vascular endothelial cells. Abbreviations: ICAM-1, intracellular adhesion molecule-1; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; TNF-α, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1.

Figure 2. Photomicrographs from confocal laser-scanning microscopy visualizing oxidative stress as DCF fluorescence emission. Endothelial cells were either untreated (left panel, control cells) or treated with 90 μM linoleic acid for 6 h (right panel). Blue color on the pseudocolor scale reflects low level of cellular oxidation, yellow intermediate, red high, and white the highest level of cellular oxidative stress. Intensity of fluorescence can be quantitated using "ImageSpace" software (Molecular Dynamics) and is expressed in relative units of DCF fluorescence.

Figure 3A. Linoleic acid (LA) treatment enhances NF-κB binding in human endothelial cells as analyzed by EMSA. Endothelial cells were either untreated (lane 2) or treated for 2 h with increasing doses of linoleic acid (lanes 4-6). Competition study was performed by the addition of excess unlabeled oligonucleotide (lane 7) using nuclear extracts from cells treated with 50 μM linoleic acid. Lane 1, probe alone; lane 3, LPS (1 μg/mL, positive control).

Figure 3B. Supershift analysis of linoleic acid (LA)-induced NF-κB binding activity in human endothelial cells. Nuclear extracts were prepared from cells treated with 50 μM linoleic acid for 2 h (lanes 2-4) and incubated with anti-p50 antibody (lane 3) or anti-p65 antibody (lane 4) for 25 min before the addition of ^{32}P-labeled probe. Lane 1, probe alone. SS indicate the bands shifted by specific antibodies.

Figure 4. Concentration-dependent upregulation of the MCP-1 mRNA expression in human endothelial cells exposed to specific dietary fatty acids. Total cDNA was synthesized from 1 μg

of cellular RNA isolated from HUVEC stimulated with 60 and 90 μM fatty acids or TNF-α (20 ng/mL, positive control) for 3h. The amplified PCR products were electrophoresed on a 2% TBE agarose gel, stained with SYBR Green I (Molecular Probes, Eugene, OR) and visualized using phosphoimaging technology (FLA-2000, Fuji, Stamford, CN). M, molecular weight markers (100-bp DNA ladder); 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid.

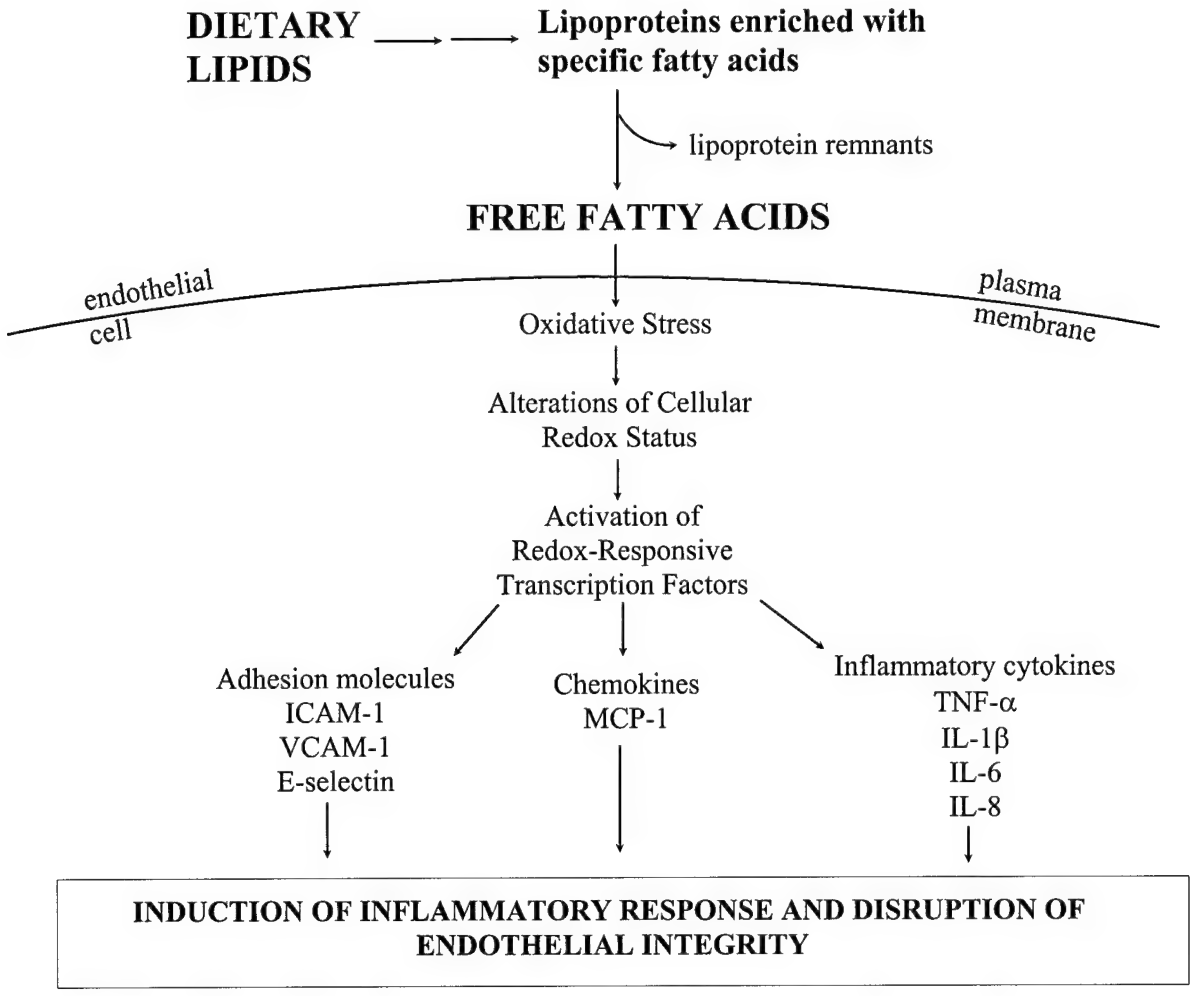
DIETARY LIPIDS
Lipoproteins enriched with specific fatty acids
lipoprotein remnants
FREE FATTY ACIDS
endothelial cell
plasma membrane
Oxidative Stress
Alterations of Cellular Redox Status
Activation of Redox-Responsive Transcription Factors
Adhesion molecules
ICAM-1
VCAM-1
E-selectin
Chemokines
MCP-1
Inflammatory cytokines
TNF-α
IL-1β
IL-6
IL-8
INDUCTION OF INFLAMMATORY RESPONSE AND DISRUPTION OF ENDOTHELIAL INTEGRITY

Figure 1

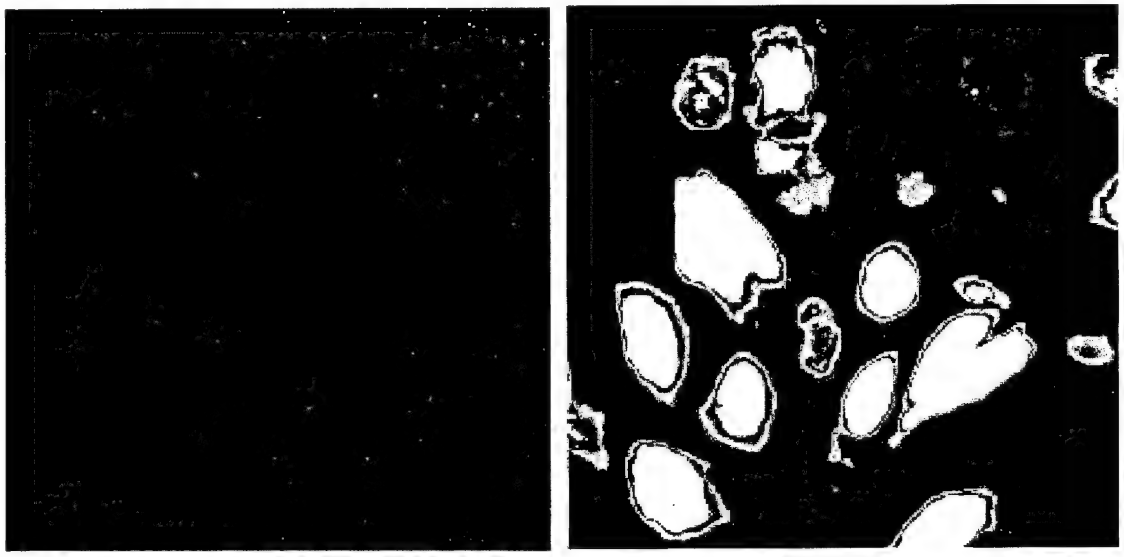

Figure 2

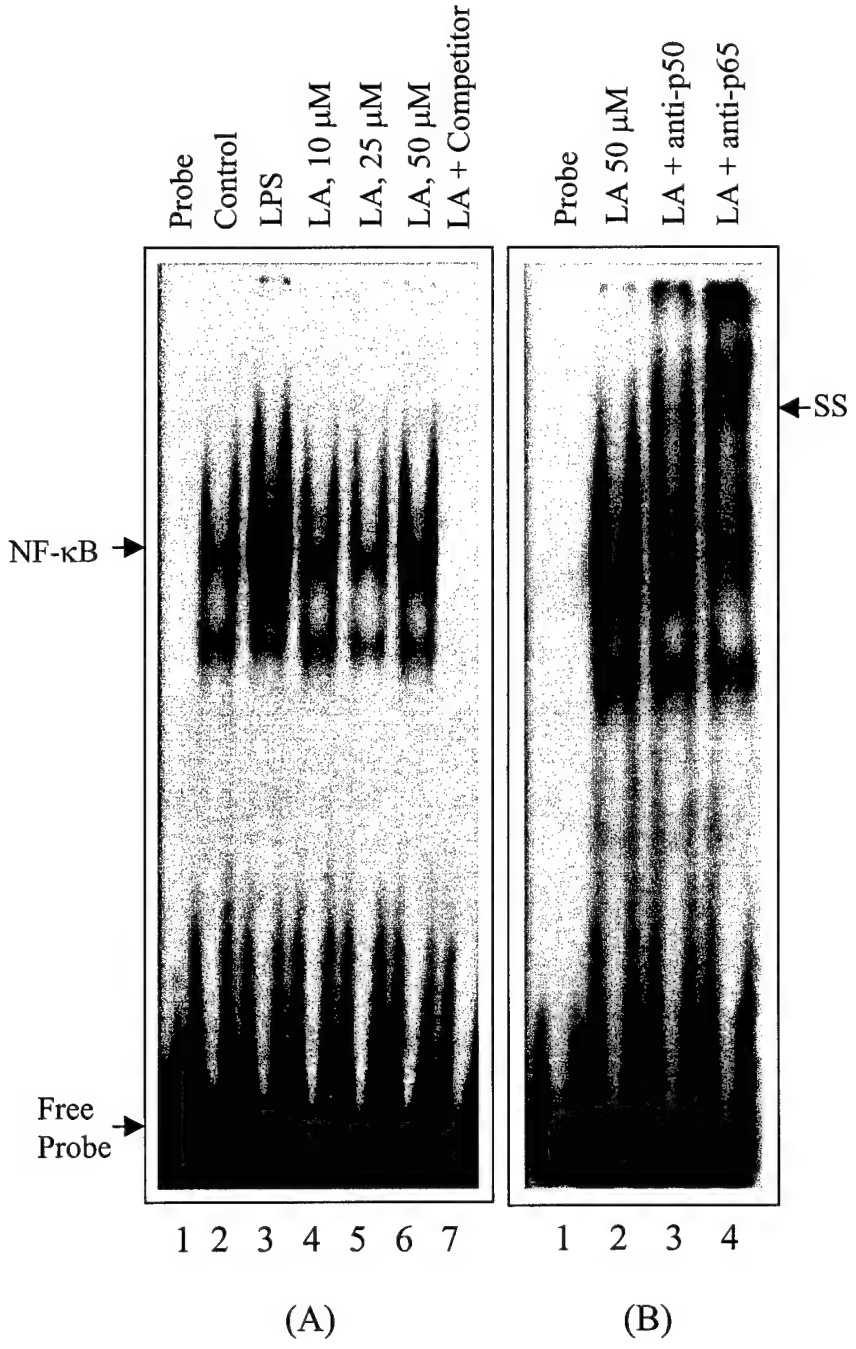
Probe
Control
LPS
LA, 10 μM
LA, 25 μM
LA, 50 μM
LA + Competitor
Probe
LA 50 μM
LA + anti-p50
LA + anti-p65
NF-κB
SS
Free
Probe
1 2 3 4 5 6 7
1 2 3 4
(A)
(B)

Figure 3

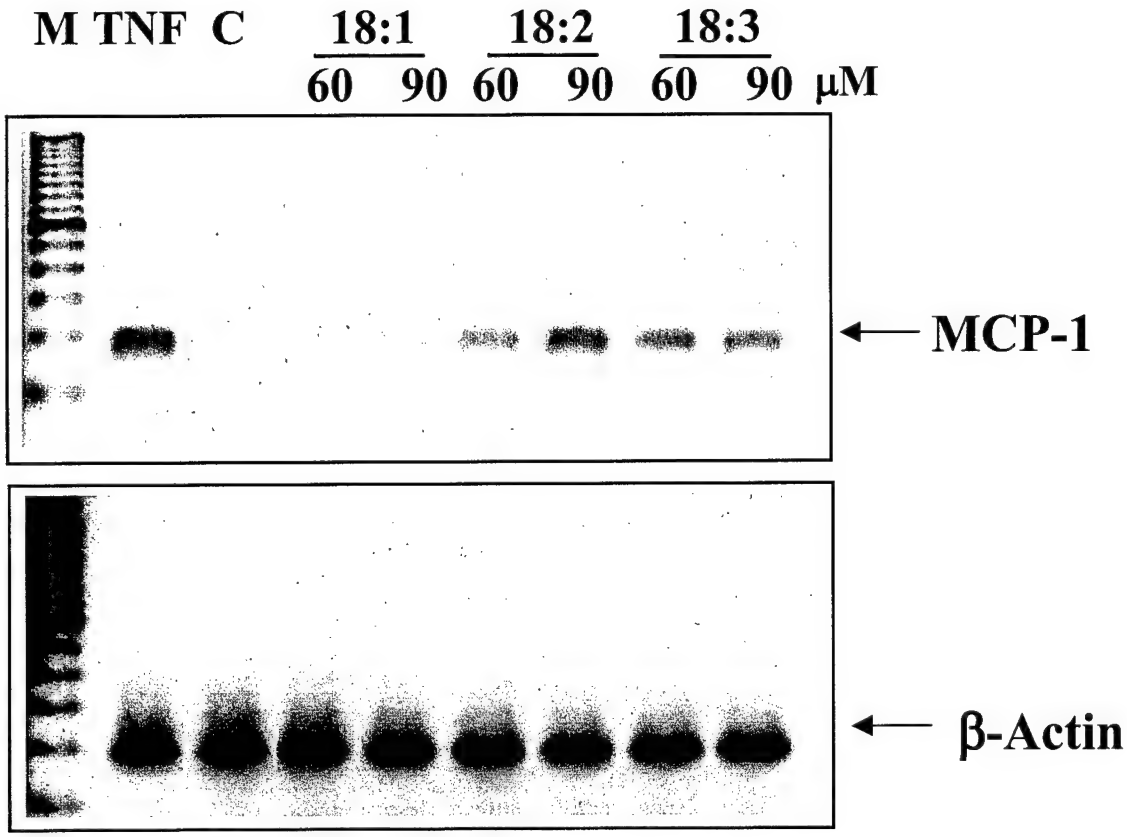

Figure 4

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Liposome-Mediated High-Efficiency Transfection of Human Endothelial Cells

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Key Words
Gene transfer · Cell culture · Vasculature

Abstract
Liposome-mediated transfection of endothelial cells provides a valuable experimental technique to study cellular gene expression and may also be adapted for gene therapy studies. However, the widely recognized disadvantage of liposome-mediated transfection is low efficiency. Therefore, studies were performed to optimize transfection techniques in human endothelial cells. The majority of the experiments were performed with primary cultures of human umbilical vein endothelial cells (HUVEC). In addition, selected experiments were performed using human brain microvascular endothelial cells and human dermal microvascular endothelial cells. To study transfection rates, HUVEC were transfected with the pGL3 vector, containing the luciferase reporter gene, complexed with several currently available liposomes, such as different Perfect Lipid (pFx) mixtures, DMRIE-C, or lipofectin. The optimal transfection rate was achieved in HUVEC transfected for 1.5 h with 5 μg/ml of DNA plasmid in the presence of 36 μg/ml of pFx-7. In addition, transfection with the VR-3301 vector encoding for human placental alkaline phosphatase revealed that, under the described conditions, transfection efficiency in HUVEC was approximately 32%. Transfections mediated by other liposomes were less efficient. The usefulness of the optimized transfection technique was confirmed in HUVEC transfected with NF-κB or AP-1-responsive constructs and stimulated with TNF or LPS. We conclude that among several currently available liposomes, pFx-7 appears to be the most suitable for transfections of cultured human endothelial cells.

Introduction

Cellular transfections (physical-chemical methods of introducing genes into cells) provide powerful experimental tools to study gene regulation in vivo and in vitro [1]. In addition, transfection techniques are used to deliver foreign DNA in gene therapy strategies [2, 3]. Stable transfections refer to the production of a population of cells in which the gene of interest is stably expressed in the cell. Thus, the gene is not only introduced into the cell but also is integrated into the host DNA and reproduced during cell cycles or cell division. The second general type of transfection is transient transfection, during which plasmid DNA is introduced into a cell population but no stable cell lines are isolated. Instead, gene expression is studied shortly after the transfection procedure, usually within 24–72 h [4]. The advantage of the second approach is the simplicity of the technique and the fact that the same

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preparation of DNA can be introduced into various cell types. Because cellular membranes create barriers for large and highly charged DNA molecules to enter cellular compartments, several techniques have been developed to facilitate cellular transfections. Transfection methods include calcium-phosphate precipitation, electroporation, detergent-DNA complexes, DNA-DEAE complexes, microinjection, virus-mediated transfection, introduction of DNA via particle bombardment and lipid-mediated transfection [2, 5]. In transfections performed in vitro in cultured cells, cationic lipids have become standard carriers of plasmid DNA [6].

Endothelial cells are a promising target in somatic gene therapy in cardiovascular disorders, ischemic disease [7] and cancer [8, 9], since the endothelium is involved in these pathological stages and endothelial cells are accessible for gene transfer via circulation [10]. Several experimental and clinical studies have demonstrated the therapeutic potential of somatic gene therapy in vascular diseases. For example, in the treatment of restenosis, positive results were obtained when animals were transfected with the genes encoding for vascular endothelial growth factor, nitric oxide synthase, thymidine kinase, retinoblastoma, growth arrest or antisense oligonucleotides against transcription factors [10, 11]. In atherosclerosis, gene therapy strategies have been used in the treatment of vascular proliferation, endothelial dysfunction, thrombosis, and ischemia as well as in modification of the blood/biomaterial interface [12]. It has also been reported that transfer of genes encoding for cyclooxygenase and endothelial nitric oxide synthase can protect against intimal hyperplasia in angioplasty-injured carotid arteries [13]. Clinical trials indicated that substantial therapeutic benefits could be obtained by intramuscular injections of naked DNA plasmid encoding for human vascular endothelial growth factor in patients with severe peripheral arterial disease [14].

The most efficient transgene expression can be achieved by using adenoviruses [15]. In fact, with adenovirus vector, recombinant genes can be delivered to approximately 100% of endothelial cells of normal human vessels in organ cultures [16]. However, adenoviral vectors can induce injury to the vessel wall. For example, in arteries transduced with replication-defective adenoviral vector AdRSVn-LacZ, a marked accumulation of macrophages and increased intimal cellularity were reported. In addition, in hypercholesterolemic cynomolgus monkeys, this vector caused an increase in vessel wall inflammation and progression of early atherosclerotic lesions [17]. Viral transduction can also induce changes in endothelial cell phenotype [18]. Therefore, nonviral transfections, including cationic liposomes, remain attractive carriers to facilitate the entry of foreign DNA into endothelial cells.

The aim of the present study was to optimize a transfection technique using different, currently available cationic liposomes in cultured human endothelial cells. Transfection rate was established using liposomes complexed with the pGL3 vector, driven by the simian virus 40 (SV40) promoter and containing the luciferase reporter gene. In addition, the efficiency of transfection was studied by employing the VR-3301 vector driven by the cytomegalovirus (CMV) promoter ligated to the human placental alkaline phosphatase reporter gene (hpAP). We found that cultured human endothelial cells can be efficiently transfected.

Materials and Methods

Endothelial Cell Cultures

Human umbilical vein endothelial cells (HUVEC) were isolated as described previously [19]. They were maintained in growth medium containing M199, 25 m*M* HEPES, 54.3 U/ml heparin, 2 m*M* *L*-glutamine, 1 μ*M* sodium pyruvate, 200 U/ml penicillin, 200 μg/ml streptomycin, 0.25 μg/ml amphotericin B (all reagents from Gibco BRL, Grand Island, N.Y., USA), 40 μg/ml endothelial cell growth supplement (ECGS, Becton Dickinson, Bedford, Mass., USA), and 20% FBS (HyClone Laboratories, Inc., Logan, Utah, USA).

Cells were determined to be endothelial by their cobblestone morphology and uptake of fluorescent labeled acetylated LDL (1,1′-dioctadecyl-3,3,3′3′-tetramethyl-indocarbocyanine perchlorate; Molecular Probes Inc., Eugene, Oreg., USA). All experiments were conducted with cells from passage two.

Selected experiments also were performed using human aortic endothelial cells (HAEC), immortalized human brain microvascular endothelial cells (HBMEC) and immortalized human dermal microvascular endothelial cells (HMEC-1). HAEC were purchased from Clonetics Corp., (Walkersville, Md., USA) and cultured in medium supplied by the manufacturer. HBMEC (a generous gift from Dr. M. Fiala, UCLA School of Medicine) were isolated from a brain biopsy of an adult female with epilepsy and immortalized by transfection with SV40 large-T antigen. They were cultured in RPMI-1640 medium (Gibco BRL), supplemented with 10% FBS (HyClone Laboratories), 10% NuSerum IV (Becton Dickinson), 1% nonessential amino acids, 1% vitamins, 5 U/ml heparin, 1 m*M* sodium pyruvate, 2 m*M* *L*-glutamine (all reagents from Gibco BRL), and 30 μg/ml ECGS (Becton Dickinson) [20, 21].

HMEC-1 (a generous gift from Dr. E. Smart, University of Kentucky) were isolated from dermal microvessels and immortalized by transfection with SV40 large-T antigen. They were cultured in MCDB-131 medium (Gibco BRL) supplemented with 10% FBS (HyClone Laboratories), 200 U/ml penicillin, 200 μg/ml streptomycin (Gibco BRL), 10 ng/ml endothelial growth factor (Calbiochem, San Diego, Calif., USA) and 1 mg/ml hydrocortisone (Sigma).

Each experiment was performed at least in triplicate on at least four (and up to 12) independent cultures.

Liposome Carriers for Transient Transfection and Transfection Procedure

The PerFect Lipid Transfection kit (Invitrogen, Carlsbad, Calif., USA), DMRIE-C, and lipofectin (Gibco BRL, Grand Island, N.Y., USA) were used for transfections of endothelial cells. The PerFect Lipid Transfection kit provides eight different compositions of lipids (pFx 1–8), and each of these lipids was employed in the present study. Molecular weights of different pFx mixtures vary from 847 (pFx-4) to 2,617 (pFx-8). The molecular weight of pFx-7, the liposome used in the majority of our experiments, is 1,011. DMRIE-C (molecular weight 646) was used because it resembles a lipid carrier which was previously used successfully for endothelial cell transfections [5]. Lipofectin (molecular weight 669.5), which was employed in our earlier study [22], is widely used in transfection of endothelial cells. Selected experiments also were performed using cytofectin GCV (Glen Research, Sterling, Va., USA), DAC-30 (Eurogentec, Sersing, Belgium), and SuperFect (Qiagen, Valencia, Calif., USA). These additional liposomes were selected based on a recent report which demonstrated that cytofectin GCV or SuperFect can mediate uptake of antisense oligonucleotides in cultured human iliac artery endothelial cells with high efficiency [23].

For transfection studies, endothelial cells were seeded in 12-well plates and grown to 50–60% confluency in normal growth medium. To perform transfections, aliquots of normal M199 were mixed with different concentrations of specific lipid carriers in polystyrene tubes, mixed with plasmid DNA and incubated at 37°C for 30 min to allow the formation of DNA-lipid complexes. Endothelial cell cultures were washed three times with M199 to remove serum, and 1 ml of transfection solution was added to each well of the 12 well plates. Controls consisted of endothelial cells incubated with plasmid DNA alone or liposomes complexed with a carrier plasmid. After incubation, transfection solutions were aspirated and replaced with growth medium. Cells were maintained in these conditions for 48 h before assays for reporter genes were performed.

pGL3 Vector and Luciferase Reporter Gene Assay

To monitor the transfection rate, endothelial cells were transfected with individual liposomes complexed with the pGL3 Luciferase Reporter Vector (Promega, Madison, Wisc., USA). This vector contains the SV40 promoter and enhancer sequence and firefly luciferase as a reporter gene. Following the transfection process, luciferase activity was measured by Luciferase Assay System (Promega) according to the instructions supplied by the manufacturer. Briefly, culture media were removed and cells were washed three times with PBS and incubated for 10 min with 60 µl of Cell Culture Lysis reagent. Attached cells were then scraped, centrifuged to remove membrane debris, transferred to new tubes, and stored at –80°C until analysis. For luciferase assay, 10 µl of the cell extracts were mixed with 100 µl of Luciferase Assay Reagent containing luciferin and ATP in a luminometer with automatic injection. Light emission was measured every 0.5 s, for 10 s. Values are expressed in RLU/µg protein. Cellular proteins were measured using Bradford reagent (Bio-Rad, Hercules, Calif., USA).

VR-3301 Vector and Alkaline Phosphatase Reporter Gene Assay

To establish transfection efficiency, endothelial cells were transfected with VR-3301 vector (Vical Inc., San Diego Calif., USA) mixed with pFx-7, DMRIE-C or lipofectin. The VR-3301 vector contains CMV promoter/enhancer which regulates expression of the hpAP gene. Transfected endothelial cells were fixed in 4% paraformaldehyde for 1 h and then washed 3 times with PBS. Following heat inactivation of endogenous alkaline phosphate isoenzymes of nonplacental origin (30 min at 65°C), cells were stained for hpAP using an azo dye coupling technique [24]. Briefly, 0.2 ml of naphtol AS-MX phosphate (0.25% alkaline solution, Sigma) were mixed with 4.8 ml of 0.1 *M* Tris-HCl buffer (pH 10.0) and 10 mg of fast red TR salt (Sigma). The stain mixture was filtered immediately before use, and cells were stained for the presence of hpAP for 15 min at room temperature. In independent sets of experiments, fluorescence of transfected cells was determined either by flow cytometry (in cell suspension) or fluorescent microscopy (in cells cultured on glass-bottom dishes) using rhodamine filter sets. Data are expressed as a percentage of cells in which activity of hpAP was detected.

Employment of the Optimized Transfection Conditions to Study Activation of Transcription Factors in Endothelial Cells

To determine whether the optimized transient transfection technique is useful in studies on transcription factor activation in endothelial cells, HUVEC were transfected for 1.5 h with 5 µg of NF-κB or AP-1 reporter plasmids (Stratagene, La Jolla, Calif., USA) mixed with 36 µg/ml of pFx-7. NF-κB responsive plasmid contained five repeats of NF-κB enhancer elements, and AP-1-responsive plasmid contained seven repeats of AP-1 enhancer elements, linked to basic TATA element and the firefly luciferase reporter gene. Following transfection, cells were incubated in normal growth medium for 24 h. Then, HUVEC were treated with either TNF (10 ng/ml) or LPS (1 µg/ml) in a medium containing 10% FBS for 24 h. At the end of the incubation time, cells were washed with PBS, lysed, and measured for luciferase activity using Luciferase Assay Reagent (Promega).

Cell Proliferation (5-Bromo-2′-Deoxyuridine Incorporation Assay)

Endothelial cell proliferation was determined by the 5-bromo-2′-deoxyuridine (BrdU) incorporation assay according to the procedure supplied by the manufacturer (Roche Diagnostics, Mannheim, Germany). This assay takes advantage of the incorporation of BrdU, instead of thymidine, into the DNA of proliferating cells. Briefly, immediately following transfections, endothelial cells were incubated for 12 h with 10 µ*M* BrdU diluted in normal growth medium. Then, cultures were fixed and incubated with monoclonal anti-BrdU antibody labeled with peroxidase. Following a 30-min incubation, tetramethylbenzidine was added as a substrate for peroxidase and, after a 10-min interval time required for color development, absorbance was read at 370 nm. The results were expressed as percentage of control.

Statistical Analysis

Statistical analysis was performed using SYSTAT 8.0 (SPPS Inc., Chicago, Ill., USA). One-way or two-way ANOVA was used to compare the mean values among the treatments. Two-way ANOVA was employed in statistical analysis of all experiments which included at least two variables, such as time and different treatment factors. When the overall F values were significant, ANOVA was followed by a posthoc Bonferroni test to compare means from different treatments. Statistical probability of $p < 0.05$ was considered significant.

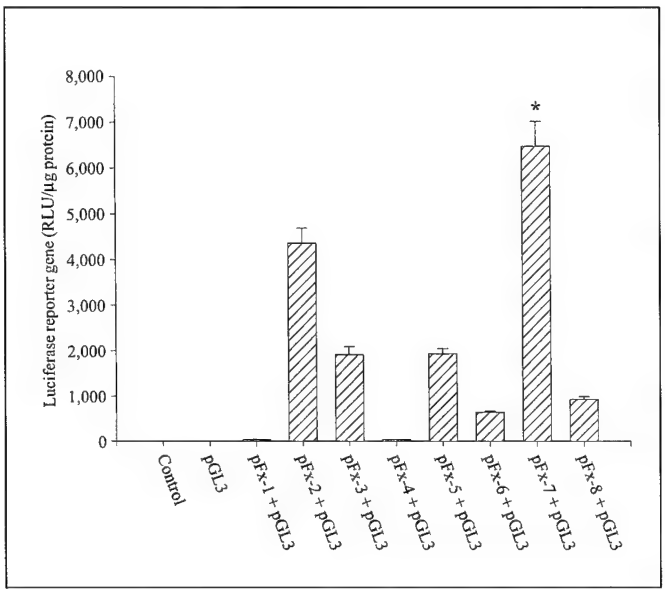


Fig. 1. Comparison of transfection rates mediated by different pFx mixtures. Cells were transfected for 3 h with 5 μg/ml of the pGL3 vector complexed with 36 μg/ml of individual pFx lipids. Transfections were followed by a 48-hour recovery period in normal growth medium, after which the reporter gene assay was performed. Values are mean ± SEM. *Values in cultures transfected by pFx-7 are significantly higher than values from groups transfected with other pFx lipids.

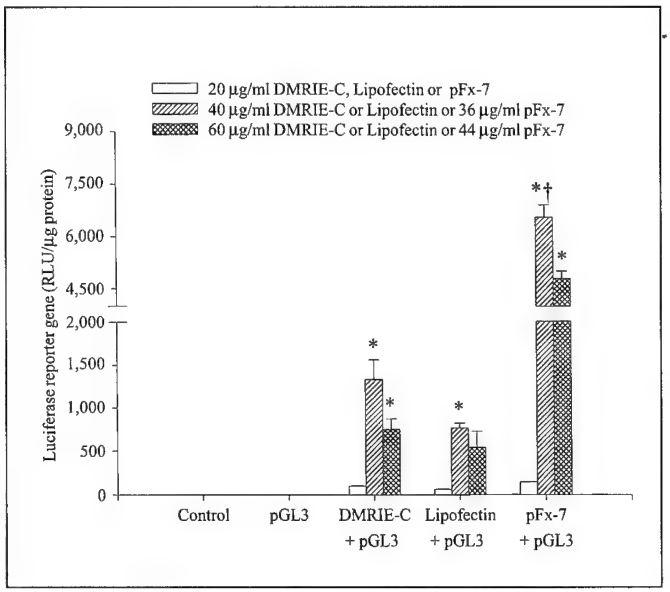


Fig. 2. The effect of liposome concentrations on transfection rates in HUVEC. Cells were transfected for 1.5 h with 5 μg/ml of the pGL3 vector complexed with different concentrations of pFx-7, DMRIE-C or lipofectin. Values are mean ± SEM. *Values are statistically significant as compared to the values in the group transfected with the preceding concentration of a given liposome. †Values in cultures transfected in the presence of 36 μg/ml pFx-7 are significantly higher than transfection rates in other experimental groups.

Results

Transient Transfection Rates Mediated by Different pFx Liposomes

To determine the most effective pFx liposome as a mediator of transient transfection of endothelial cells, HUVEC were transfected with 5 μg/ml of the pGL3 vector complexed with 36 μg/ml of each liposome provided in the PerFect Lipid Transfection kit. Figure 1 indicates transfection rates, as determined by luciferase activity, mediated by individual pFx liposomes. Transfections were performed for 3 h, followed by a 48-hour recovery process. Only minimal transfection rates (range of 3–7 RLU/μg protein) were determined in HUVEC exposed to the pGL3 vector alone. Except for pFx-1 and pFx-4, all remaining pFx liposomes successfully mediated transfection of HUVEC. However, the most marked transfection rate was observed in cells transfected with pFx-7. Therefore, optimization of transfection conditions was performed with this liposome.

Comparison of Transfection Rates Mediated by DMRIE-C, Lipofectin, or pFx-7 and Optimization of Liposome Concentrations

DMRIE-C reagent and lipofectin are commercially available liposomes, widely used to initiate transient or stable transfections. To establish the most suitable liposome carrier and the optimal liposome concentration for transient transfection of endothelial cells, the pGL3 vector (5 μg/ml) was complexed with different concentrations of DMRIE-C, lipofectin or pFx-7. Transfections were performed for 1.5 h, followed by a 48-hour recovery period. Results of these experiments are reflected in figure 2. Liposomes at the concentrations of 20 μg/ml (or lower – data not shown) appeared to be ineffective in HUVEC transfection. However, an increase in liposome concentrations from 20 to 40 μg/ml for DMRIE-C or lipofectin and to 36 μg/ml for pFx-7 resulted in an increase of transfection rates, as measured by luciferase activity. In particular, a dramatic increase (approximately 250 times) in transfection rate was detected in HUVEC transfected with pGL3 complexed with pFx-7 at the concentration of 36 μg/ml. The rate of transfection mediated by this con-

centration of pFx-7 was approximately 8.5 times higher compared to transfection induced by 40 μg/ml lipofectin. In addition, the transfection rate achieved by pFx-7 exceeded that mediated by 40 μg/ml DMRIE-C by almost 5 times. Further increase in concentrations of DMRIE-C, lipofectin or pFx-7 decreased transfection rates. It appears that a marked cytotoxicity observed in endothelial cell cultures exposed to high doses of liposomes was responsible for this phenomenon.

In separate experiments, transfection rates mediated by pFx-7 at the dose of 36 μg/ml were compared to those mediated by cytofectin GCV (used at the concentration range of 1–40 μg/ml), DAC-30 (concentration range of 5–30 μg/ml), and SuperFect (concentration range of 20–80 μg/ml). Among these liposomes, transfection of HUVEC mediated by pFx-7 also resulted in the highest transfection rates (data not shown).

Liposome-Mediated Toxicity in Cultured Endothelial Cells

When introduced into cell cultures, liposomes can induce cytotoxic effects which depend on lipid concentration and transfection time. Therefore, their toxic effects were also measured in cultured endothelial cells. BrdU incorporation assay, which reflects cell proliferation, was used in these studies. As indicated in table 1, treatments with lipofectin appeared to be most toxic in cultured endothelial cells. Diminished incorporation of BrdU was observed in endothelial cells incubated with 40 or 60 μg/ml of lipofectin for as short as 1 h. In addition, when cells were treated with lipofectin for 3 h, even lower doses of this liposome decreased proliferation of endothelial cells.

Transfection mediated by pFx-7 resulted in a moderate toxicity. Endothelial cell proliferation was not statistically decreased when this liposome was used at the doses of up to 36 μg/ml for 1 or 1.5 h. However, a higher dose (i.e., 44 μg/ml) of pFx-7 as well as a 3 h incubation time markedly diminished incorporation of BrdU in transfected HUVEC (table 1). In general, the most marked cytotoxicity was observed when endothelial cells were exposed to high doses of liposomes for 3 h. Liposome-mediated toxic effects similar to those detected in HUVEC were observed in cultures of HAEC (data not shown).

Although 1.5-hour treatments with liposomes at concentrations which mediated the optimal transfection rates as reported in figure 2 did not affect BrdU incorporation, they resulted in morphological changes of cultured endothelial cells. Because the character of these changes was similar for all studied liposomes, they are documented only for pFx-7, the liposome which produced the highest transfection rates in HUVEC. Figure 3A reflects morphological alterations of HUVEC, as observed under a phase-contrast microscope, after a 1.5-hour incubation with 36 μg/ml pFx-7 complexed with 5 μg/ml of the pGL3 vector. Cytotoxic effects of this complex included cellular shrinkage and detachment. However, a 48-hour recovery period following transfection, during which cells were maintained in normal growth medium, allowed HUVEC to regain normal morphological features. This phenomenon is shown in figure 3B, a photograph of the same culture as depicted in figure 3A, but taken after the recovery period.

Table 1. Toxic effects of different transfection carriers as measured by the incorporation of BrdU assay

Transfection carrier	Exposure time		
	1 h	1.5 h	3 h
Lipofectin, μg/ml			
10	98.7±3.94	94.1±1.97	69.9±0.82[a,b]
20	97.1±4.70	91.7±2.57	59.4±4.75[a,b]
40	79.4±8.49[a]	74.1±4.96[a,c]	47.3±4.69[a,b]
60	60.9±4.06[a]	54.3±0.33[a,c]	47.4±5.47[a]
DMRIE-C, μg/ml			
10	98.5±6.37	90.5±1.31	96.6±2.10
20	105.2±4.45	87.3±7.32	76.2±1.47[a,c]
40	101.7±2.69	86.1±1.38[a]	74.4±3.17[a,b]
60	102.4±1.22	81.2±2.01[a,b]	64.7±2.55[a,b]
pFx-7, μg/ml			
12	98.4±3.30	92.2±3.07	93.5±4.28
24	95.1±2.63	86.9±2.30	85.6±3.22
36	88.7±5.82	84.2±4.79	66.7±0.98[a,c]
44	65.0±3.11[a,c]	61.5±4.94[a–c]	52.6±2.69[a,c]

Values are mean ± SEM and are expressed as percentage of control.

[a] Statistically different as compared to control, i.e., non-transfected cells.

[b] Statistically different as compared to the values in the group transfected with the same concentration of a given liposome for the preceding exposure time.

[c] Statistically different as compared to the values in the group transfected for the same exposure time with the preceding concentration of a given liposome.

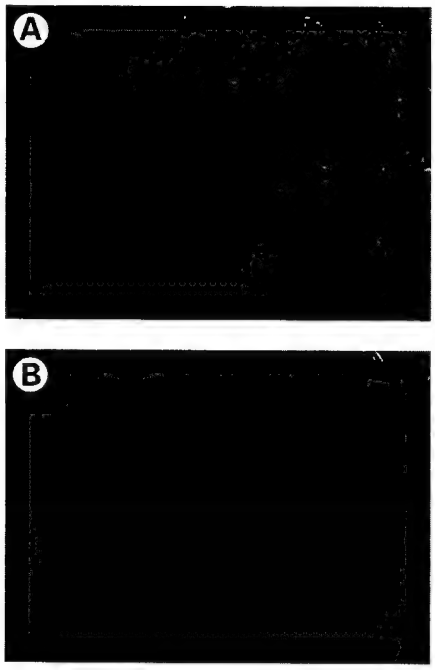


Fig. 3. The effect of pFx-7-mediated transfection on HUVEC morphology as observed under a phase-contrast microscope. Cells were transfected for 1.5 h with the pGL3 vector complexed with 36 μg/ml of pFx-7. **A** Cell morphology at the end of the 1.5-hour transfection period. **B** Cell morphology at the end of the 48 hour recovery period in which cells were maintained in normal medium.

Optimization of Plasmid DNA Concentration and Transfection Time for Transient Transfection of Endothelial Cells

Both the amount of plasmid DNA used for transfection and transfection time are important factors which can determine the transfection rate. Figure 4 shows transfection rates in HUVEC transfected with different amounts of the pGL3 vector complexed with pFx-7 at the concentration of 36 μg/ml as well as with DMRIE-C or lipofectin at the concentration of 40 μg/ml. Maximum transfection rate was observed in cells transfected with 5 μg/ml of plasmid DNA. In cells transfected with either 2 or 10 μg pGL3/ml, transfection rates were minimal as compared to 5 μg/ml of the pGL3 vector.

To determine the optimal transfection time, HUVEC were transfected for 1, 1.5 or 3 h, followed by a 48-hour recovery period. Time-dependent effects on liposome-mediated transfection are also shown in figure 4. As reflected in this figure, most successful HUVEC transfections resulted from 1.5-hour transfection time. Transfection rates in cells exposed to liposomes for that period of time were constantly higher than those in HUVEC transfected for 1 h. In addition, extension of transfection time to 3 h did not result in higher transfection rates. It appears that cytotoxicity of liposomes, as reported in table 1, could affect transfection rates in HUVEC transfected for 3 h.

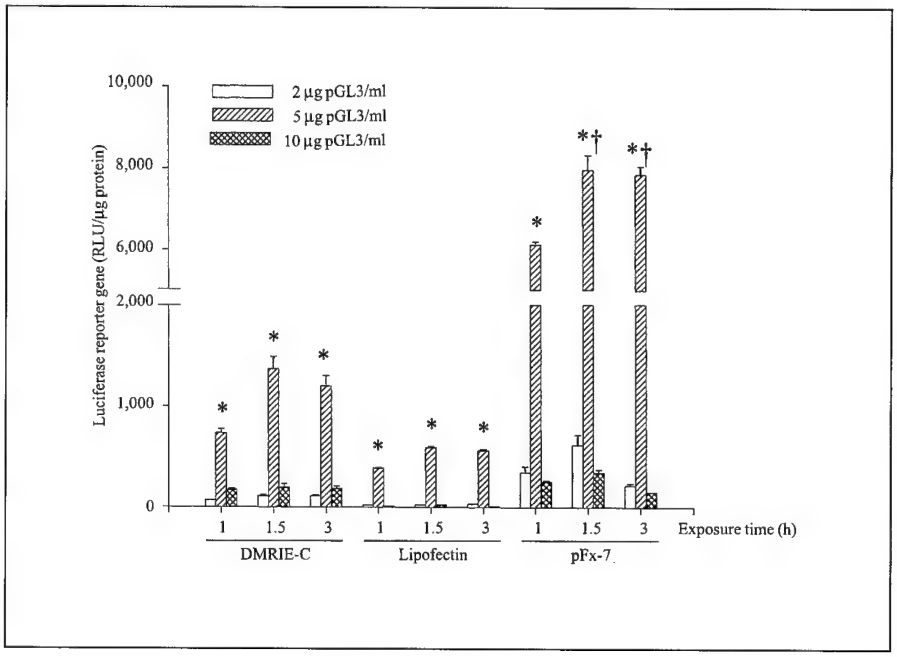


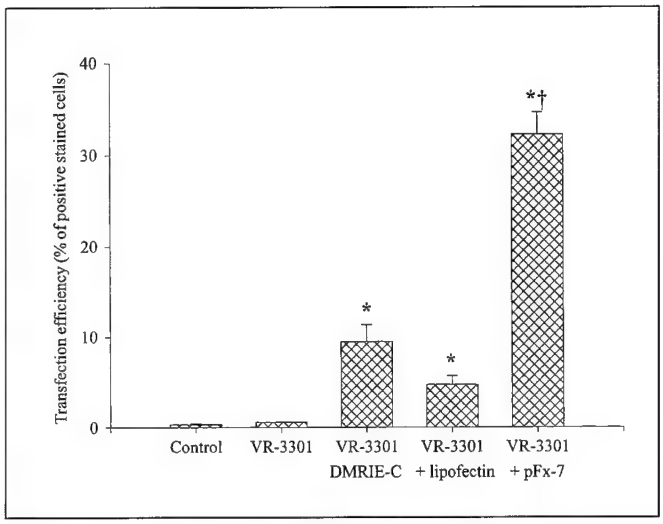
Fig. 4. Comparison of transfection rates mediated by DMRIE-C, lipofectin, or pFx-7 under different concentrations of plasmid DNA and transfection times. HUVEC were transfected for 1, 1.5 or 3 h with different concentrations of the pGL3 vector complexed with DMRIE-C or lipofectin at the concentration of 40 μg/ml or with pFx-7 at the concentration of 36 μg/ml. Values are mean ± SEM. Luciferase activities in control (nontransfected) cells and in cells transfected with naked pGL3 were negligible and were not plotted. *Values in cultures transfected with 5 μg pGL3/ml are significantly higher than values from groups transfected with other amounts of plasmid DNA. †Values in cultures transfected for 1.5 or 3 h in the presence of 36 μg pFx-7/ml complexed with 5 μg pGL3/ml are significantly higher than transfection rates in other experimental groups.

Efficiency of Transient Transfection in Endothelial Cells

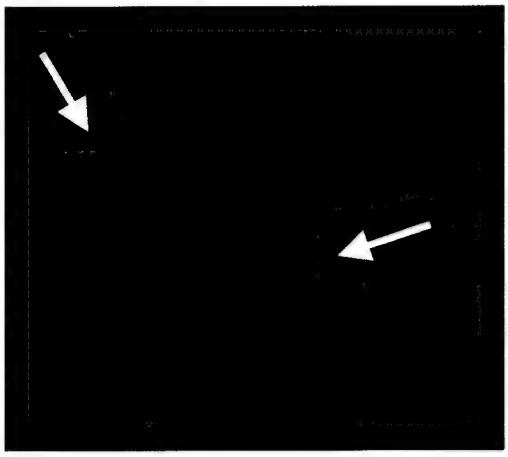
Previously described experiments allowed us to determine the optimal transfection conditions for HUVEC using individual liposomes , i.e., pFx-7 at the concentration of 36 μg/ml, DMRIE-C or lipofectin at the concentration of 40 μg/ml, a transfection time of 1.5 h, and plasmid DNA concentration of 5 μg/ml. Using these experimental settings, transfection efficiency was measured by determination of activity of human placental alkaline phosphatase (hpAP) in HUVEC transfected with the VR-3301 vector, encoding for hpAP, and complexed with pFx-7, DMRIE-C or lipofectin. A fluorescent marker of hpAP activity, the fast red TR salt, was employed in these studies, and fluorescence was measured by either flow cytometry (in cell suspension) or fluorescent microscopy. Figure 5A shows the results of the quantitative analysis of transfection efficiency performed by flow cytometry. Under the described conditions, transfection efficiency in HUVEC mediated by pFx-7 was determined to be 34.4%. In contrast, transfection efficiency in endothelial cells transfected with DMRIE-C or lipofectin was much lower, i.e. approximately 9.5 or 4.7%, respectively. In control cultures and in cultures exposed to the naked plasmid DNA, positive staining for hpAP was negligible. Figure 5B depicts HUVEC positively stained for the presence of alkaline phosphatase (arrows) as observed under the fluorescent microscope.

Effectiveness of the Optimized Transfection Technique to Study Activation of Transcription Factors in HUVEC and for Transient Transfection of Different Endothelial Cell Types

One of the major applications of transient transfections is to study activation of transcription factors and mechanisms of gene regulation. Therefore, our optimized transfection technique (i.e., pFx-7, 36 μg/ml; plasmid DNA concentration, 5 μg/ml; transfection time, 1.5 h followed by a 48-hour recovery period) was employed in such an experimental setting. HUVEC were transfected with NF-κB- or AP-1-responsive plasmids containing the firefly luciferase reporter gene, and luciferase activity was determined in cells stimulated with TNF (10 ng/ml) or LPS (1 μg/ml). The results of these experiments are shown in figure 6. Both TNF and LPS significantly increased luciferase activity in HUVEC transfected with NF-κB or AP-1-responsive plasmids. These data are consistent with TNF or LPS-induced activation of NF-κB or AP-1 in HUVEC, as determined by electrophoretic mobility shift assay (data not shown).



A



B

Fig. 5. A Efficiency of liposome-mediated transfection in HUVEC as measured by flow cytometry. Cells were transfected for 1.5 h with the VR-3301 vector (5 μg/ml) complexed with 40 μg/ml of DMRIE-C or lipofectin or with 36 μg/ml of pFx-7. *Values marked with an asterisk are significantly higher as compared to the values for control cultures or cultures transfected with naked plasmid DNA. †Values in cultures transfected in the presence of pFx-7 are significantly higher than values in other experimental groups. **B** An example of HUVEC positively stained for hpAP as observed under a fluorescent microscope (rhodamine filter). Transfection was mediated by pFx-7 under conditions as described in the legend to **A**.

Structure and functions of endothelial cells originated from different tissues differ markedly [25]. Therefore, experiments were performed in which the optimized transfection technique was employed to compare transfection rates in different types of endothelial cells, namely in HUVEC, HAEC, HBMEC, and HMEC-1. The optimized transfection conditions (i.e., pFx-7, 36 μg/ml; pGL3,

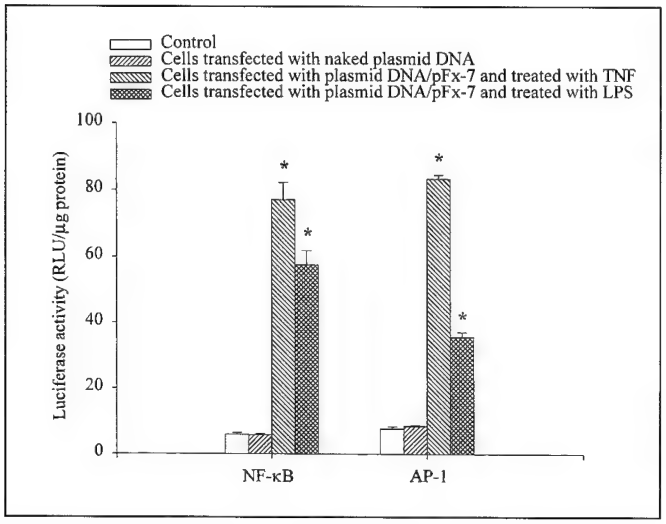


Fig. 6. The effectiveness of the optimized transfection conditions to study activation of transcription factors in HUVEC. Cells were transfected for 1.5 h with 5 μg/ml of the NF-κB or AP-1-responsive constructs complexed with 36 μg/ml of pFx-7. Transfections were followed by a 24-hour recovery period in normal growth medium, after which cells were treated either with TNF-α (10 ng/ml) or LPS (1 μg/ml) for 24. Values are mean ± SEM. *Values marked with an asterisk are significantly higher as compared to those of control cultures or cultures transfected with naked plasmid DNA.

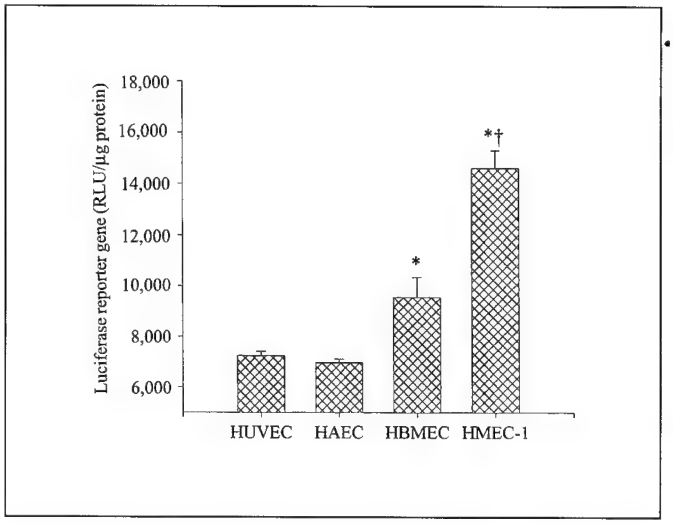


Fig. 7. A comparison of transfection rates in different types of human endothelial cells. HUVEC, HAEC, HBMEC, and HMEC-1 were transfected for 1.5 h with 5 μg/ml of the pGL3 vector complexed with 36 μg/ml of pFx-7. Luciferase activities in control (non-transfected) cells and in cells transfected with naked pGL3 were negligible and were not plotted. Values are mean ± SEM. *Transfection rates in HBMEC and HMEC-1 are significantly higher than those in HUVEC. †Transfection rates in HMEC-1 are significantly higher than those in other experimental groups.

5 μg/ml; transfection time, 1.5 h followed by a 48-hour recovery period) were employed in these experiments. As depicted in figure 7, among studied endothelial cell types, pFx-7 mediated the highest transfection rates in the immortalized endothelial cell lines, in particular in HMEC-1. There were no differences in transfection rates between HUVEC and HAEC.

Discussion

Cationic liposomes are positively charged lipids which can be mixed with negatively charged DNA to form lipid-DNA complexes. The most important advantages of mediating transfection with liposomes are that they are easy to prepare, they can transfer genes of various sizes and they are not infectious [6]. The most recognized disadvantage of liposome-mediated transfection is low efficiency of transfection. However, it is possible that the development of new generations of cationic lipids and transfection methods may overcome this limitation. In addition, better understanding of the mechanisms of liposome-mediated transfection may also contribute to the development of experimental methods which would allow for higher transfection efficiency [26].

Several factors can affect liposome-mediated transfection, including cell type, culture conditions, lipid composition of the liposomes, promoter type, reporter gene type, and amount of transfected plasmid DNA and DNA/lipid ratio. The dependency of transfection on the type of endothelial cells was observed in the present study. In the present study, we observed that transfection rates in HUVEC were approximately at the same rate as in HAEC but significantly lower as compared to immortalized endothelial cell lines (fig. 7). This is in agreement with a widely accepted phenomenon that cell lines are easier to transfect than primary cell cultures, such as HUVEC. However, it should be noted that endothelial cells, in general, are difficult to transfect. This may relate to the fact that endothelial cells represent a physiologic barrier against invasion of the vessels and underlying tissues by exogenous substances. During liposome-mediated transfection, lipids can fuse with cell membranes and thus deliver DNA into the cytoplasm. Liposome-mediated transfections are usually more efficient in dividing cells, because the nuclear membrane, which prevents DNA

from entering the nucleus, is not present during replication [6]. For this reason, transfections performed in the present study were initiated at approximately 55–65% confluency, i.e., in a state when cultured endothelial cells divide rapidly. However, it should be pointed out that liposomes can also transfect non-replicating cells [2].

Although liposome-delivered foreign DNA can enter the nucleus, it is not incorporated into the host genome. Therefore, liposome-mediated transfections are not mutagenic. The transfected plasmids remain as episomal nonreplicating minichromosomes and are gradually degraded [4]. In the present study, the reporter gene assays were performed 48 h following transfection, the standard interval for measuring reporter gene expression in cell cultures [4].

Because of the heterogeneity of cellular membranes, for optimal transfection, different types of cells require liposomes characterized by specific lipid profiles. In fact, lipid composition is the most critical factor determining the efficiency of liposome-mediated transfection. In the present study it was determined that among several commercially available liposomes pFx-7 is the most suitable lipid carrier for transfection of HUVEC. The optimal transfection rate was achieved when cells were incubated for 1.5 h with 36 μg pFx-7/ml complexed with 5 μg of plasmid DNA (fig. 2, 4). Although relatively high concentrations of pFx-7 induced cytotoxic effects in HUVEC, maintaining cells in normal growth medium for 48 h following transfection allowed for full recovery of morphological features (fig. 3). Among studied liposomes, incubation of endothelial cells with lipofectin resulted in most marked inhibition of endothelial cell proliferation (table 1). This is in agreement with an earlier report in which high toxicity of this liposome also was observed in cultured human endothelial cells [23].

In addition to comparing transfection efficiency in HUVEC mediated by different liposomes, transfections with the pGL3 vector alone were also included in the present study. It has been reported that injection with naked DNA plasmid encoding for VEGF into skeletal muscle was beneficial in patients with critical limb ischemia [14]. In addition, exposure of neurons to naked decoy κB DNA inhibited amyloid β-peptide-induced NF-κB activation [27]. However, in the present study transfection of HUVEC with naked DNA produced only a minimal effect. This is in agreement with the earlier report in which transfection efficiency with naked DNA plasmid was reported as low as approximately 0.08% [5].

Rates of liposome-mediated transfection are dependent on amounts of plasmid DNA and thus on the ratio of DNA/cationic lipids. Our studies revealed that the transfection rate of HUVEC can be enhanced with an increase in the amount of transfected DNA up to 5 μg DNA/ml (fig. 4). Further increases in the amount of plasmid DNA, and thus alteration of the DNA/liposome ratio, decreased efficiency of transfection. Similar results were obtained in the earlier studies [5]. Therefore, 5 μg DNA/ml was the standard amount of plasmid DNA used in the majority of the reported experiments.

The type of promoter which regulates the transgene expression can greatly influence transfection efficiency [28]. For example, using a plasmid regulated by the human β-actin promoter, it was reported that efficiency of transfection of HUVEC by electroporation was approximately 0.68%, by lipofectin approximately 0.45%, and by other transfection methods, including calcium phosphate and DEAE-dextran-mediated transfection, also below 1% [29]. In contrast, lipofectin-mediated transfection of HUVEC with a plasmid regulated by a strong respiratory syncytial virus (RSV) viral promoter resulted in transfection efficiency as high as 10–20% [30]. Highly efficient transfection of approximately 20% was also achieved in HUVEC transfected with a plasmid regulated by the CMV promoter, using γAP-DLRIE/DOPE liposomes [5]. Constructs employed in the present study also contained strong promoters. The pGL3 vector is regulated by the SV40 promoter and the VR-3301 vector contains the CMV promoter. Because these strong promoters use transcription factors which are present in host cells, they can be constitutively active in transfected cells. For example, the CMV promoter contains binding elements for common transcription factors, such as cyclic adenosine monophosphate and NF-κB [2]. These transcription factors remain active at the baseline level even in non-stimulated cells. In addition, one may suggest that cellular stress connected with transfection may further stimulate activation of these transcription factors. It should be noted that the CMV promoter can provide better transfection rates in HUVEC compared to the RSV promoter. This was demonstrated in experiments in which HUVEC were transfected with plasmids encoding for the same reporter gene (hpAP) but driven either by the CMV or the RSV promoter [5].

In the present study, transfection conditions were optimized using the pGL3 vector regulated by the SV40 promoter and encoding for firefly luciferase. Firefly luciferase has been recognized to be the reporter gene of choice for transfection studies in cells resistant to uptake of foreign DNA [31]. The transgene is simple to measure and has no background levels in animal tissues. In contrast,

our preliminary experiments with β-galactosidase revealed background activity of this enzyme in cultured HUVEC (data not shown). Determination of luciferase activity also has the advantage of being several orders of magnitude more sensitive than other common reporter gene assays, such as activities of chloramphenicol acetyltransferase, β-galactosidase or alkaline phosphatase [4, 31]. However, to determine the efficiency of transfection, the vector encoding for hpAP was used. This experimental approach allowed us to stain and count the transfected cells. Transfection efficiency of 32% achieved in HUVEC in the present study is higher than in earlier studies which reported efficiencies of approximately 20% [5, 30]. However, it should be noted that much higher transfection efficiency can be achieved for liposome-mediated transfection of endothelial cells with antisense oligonucleotides. For example, it was reported that cytofectin GCV or SuperFect can mediate the uptake of antisense oligonucleotides to more than 95% of cultured human iliac artery endothelial cells [23]. In contrast, these liposomes appeared to be less effective in facilitation of transfection of plasmids, such as the pGL3 vector, into HUVEC (data not shown).

In the present study, a strong correlation between transfection rates and transfection efficiency was observed. The high transfection rates mediated by pFx-7 were associated with high transfection efficiency in endothelial cells transfected in the presence of this liposome. In contrast, transfections mediated by either DMRIE-C or lipofectin resulted in moderate transfection rates and efficiency.

In summary, efficient transfection conditions have been established for a transient transfection of human endothelial cells. The optimal transfection conditions, resulting in the transfection efficiency of approximately 32%, were achieved with cationic liposome pFx-7 used at the concentration of 36 μg/ml for 1.5 h. Although these transfection conditions were connected with some cytotoxicity, a 48-hour period of maintaining endothelial cells in normal growth medium allowed the cells to recover fully. We conclude that pFx-7 can be used as an efficient transfection agent to deliver foreign DNA into human endothelial cells.

Acknowledgements

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Fatty Acid–Mediated Activation of Vascular Endothelial Cells

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Vascular endothelial cell activation and dysfunction are critical early events in atherosclerosis. Selected dietary lipids (eg, fatty acids) may be atherogenic by activating endothelial cells and by potentiating an inflammatory response. Due to their prooxidant property, unsaturated fatty acids may play a critical role in endothelial cell activation and injury. To test this hypothesis, porcine endothelial cells were exposed to 18-carbon fatty acids differing in the degree of unsaturation, ie, 90 μmol/L stearic (18:0), oleic (18:1n-9), linoleic (18:2n-6), or linolenic acid (18:3n-3) for 6 to 24 hours and/or tumor necrosis factor alpha ([TNF-α] 500 U/L) for up to 3 hours. Compared with control cultures, treatment with 18:0 and 18:2 decreased glutathione levels, suggesting an increase in cellular oxidative stress. Both 18:2 and 18:0 activated the transcription factor nuclear factor κB (NF-κB) the most and 18:1 the least. This NF-κB–dependent transcription was confirmed in endothelial cells by luciferase reporter gene assay. The fatty acid–mediated activation of NF-κB was blocked by preenrichment of the cultures with 25 μmol/L vitamin E. All fatty acids except 18:1 and 18:3 increased transendothelial albumin transfer, and 18:2 caused the most marked disruption of endothelial integrity. Preenrichment of endothelial cells with 18:2 followed by exposure to TNF-α resulted in a 100% increase in interleukin-6 (IL-6) production compared with TNF-α exposure alone. In contrast, cellular preenrichment with 18:0, 18:1, or 18:3 had no effect on TNF-α–mediated production of IL-6. Cellular release of radiolabeled arachidonic acid (20:4) was markedly increased only by cell exposure to 18:2 and 18:3, and the release of 20:4 appeared to be mainly from the phosphatidylethanolamine fraction. These data suggest that oleic acid does not activate endothelial cells. Furthermore, linoleic acid and other omega-6 fatty acids appear to be the most proinflammatory and possibly atherogenic fatty acids.

EVIDENCE SUGGESTS that the mechanisms of vascular disease such as atherosclerosis involve damage to the endothelium, which then reduces its effectiveness as a selectively permeable barrier to plasma components.[1,2] The endothelium interacts with the blood and underlying tissues, serves as both a prothrombotic and antithrombotic surface, and releases regulatory factors important in modulating vascular tone. Factors implicated in the pathogenesis of atherosclerosis include chronic and cumulative metabolic alterations of the endothelium induced by numerous activating molecules, such as certain lipids, prooxidants, and inflammatory cytokines. These risk factors may contribute to an overall cellular imbalance of the oxidative stress/antioxidant balance, thus leading to chronic activation or stimulation of the endothelium, as well as endothelial barrier dysfunction, which can result in accelerated uptake of cholesterol-rich lipoproteins into the vessel wall.

There is ample evidence suggesting that serum cholesterol is a predictor of atherosclerosis and that serum cholesterol concentrations can be modified by varying the composition of dietary fat. However, less is known about the role of specific fatty acids in atherosclerosis. The role of saturated fatty acids in atherosclerosis has been questioned recently.[3-5] In fact, data obtained in subjects with varying degrees of coronary atherosclerosis support the hypothesis that high serum polyunsaturated fatty acid levels (eg, linoleic acid), when insufficiently protected by antioxidants (eg, vitamin E), may indicate a higher risk of atherosclerosis.[6] Recent research with a population from a country with one of the highest dietary polyunsaturated to saturated fat ratios in the world has concluded that diets rich in n-6 (or omega-6) fatty acids may contribute to an increased incidence of atherosclerosis, hyperinsulinemia, and tumorigenesis.[7]

A transcription factor implicated in many endothelial cell activation responses to injury and stress is nuclear factor κB (NF-κB).[8,9] NF-κB plays a central role in regulating the cytokine network, and hence its activation may be a major factor in the pathogenesis of atherosclerosis. NF-κB can be activated by a variety of pathogenic or pathogen-elicited stimuli including cytokines, lipids, mitogens, bacteria, and related products, with the common denominator apparently being reactive oxygen species. Many target genes in endothelial cells contain NF-κB or NF-κB–like binding sites in the promoter genes coding for inflammatory cytokines (eg, tumor necrosis factor [TNF] and interleukin-6 [IL-6] and adhesion molecules).[10]

In light of the evidence that oxidative stress plays a critical role in atherosclerosis[11,12] and that antioxidant nutrients such as vitamin E may provide protection against this disease,[13,14] one may speculate that the atherosclerotic risk of dietary lipids may be directly related to their degree of unsaturation. Thus, a focus of the present study was to examine the mechanisms of the effects of 18-carbon fatty acids, differing in degree of unsaturation, on endothelial cell activation.

MATERIALS AND METHODS

Cell Culture and Experimental Media

Porcine pulmonary artery–derived endothelial cells were isolated from porcine pulmonary arteries and cultured as described previously.[15]

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Cells were subcultured in medium 199 (M-199) containing 10% bovine calf serum (HyClone Laboratories, Logan, UT) using standard techniques. The purity of the cultures was determined by morphological criteria and by quantitatively measuring angiotensin-converting enzyme activity, or by the uptake of fluorescent-labeled acetylated low-density lipoprotein (1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate; Molecular Probes, Eugene, OR).

The experimental media were composed of M-199 enriched with 5% FBS and either fatty acids (90 μmol/L) or TNF-α (500 U/mL or 100 ng/mL; Knoll Laboratories, Whippany, NJ). Fatty acids (>99% pure) were obtained from Nu-Chek Prep (Elysian, MN). Preparations of experimental media with fatty acids and/or TNF were made as described previously.[15,16] Thus, fatty acids were introduced into the media bound to serum albumin. Assuming albumin concentrations of 30 μmol/L (in 5% serum) to 60 μmol/L (in 10% serum) in our culture media, the fatty acid concentrations are within physiological and metabolic relevance. Even though only about 5% of total free fatty acids in the experimental media are derived from the serum, fatty acid–mediated activation of endothelial cells may vary depending on the type of serum in which cells are cultured.[17] For most experimental settings, cells were treated with fatty acids for 6 to 24 hours and/or TNF for 3 hours before termination. Some cultures were preenriched with 25 μmol/L vitamin E (α-tocopherol). All experimental outcomes were confirmed more than twice.

Glutathione Assay

Glutathione assays were performed according to a modified method of Baker et al.[18] To determine total glutathione, cellular protein was precipitated by adding 100 μL ice-cold 0.09% sulfosalicylic acid (SSA) to cells collected from P-100 tissue culture plates. The culture plates were then incubated at 40°C for 15 minutes, after which the cell lysates were collected and centrifuged at 9,000× *g* for 5 minutes. Glutathione levels were determined spectrophotometrically using the glutathione-linked 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) recycling assay. The mixture for the assay contained 50 μL supernatant and 100 μL 125-mmol/L phosphate buffer containing 0.225 mmol/L DTNB, 0.302 mmol/L NADPH, and glutathione reductase at a concentration of 1.25 U/μL. The blank contained 50 μL 0.09% 5-SSA instead of the supernatant, and the control reaction contained the glutathione standard in place of the supernatant. The mixtures were equilibrated at room temperature for 3 minutes, and the reaction was started by the addition of 100 μL reaction buffer. Absorbance was measured at 405 nm in a 96-well plate reader.

Transcription Factor (NF-κB) Activation Studies: Electrophoretic Mobility Shift Assay

These transcription factors, which bind to enhancer elements on DNA, were determined in endothelial cells by an electrophoretic mobility shift assay as described by Sen and Baltimore.[19] Nuclear extracts containing the NF-κB active protein were prepared from cells according to the method of Dignam et al.[20] Nuclear extracts were incubated for 20 to 30 minutes with ^{32}P-end-labeled oligonucleotide probe (GIBCO/BRL, Gaithersburg, MD) containing the κB enhancer DNA element with a tandem duplicate of a NF-κB binding site (-GGGGACTTTCC-). Incubation at room temperature was performed in the presence of nonspecific competitor DNA. Following binding, the complexed and uncomplexed DNA in the mixture were resolved by electrophoresis in a 5% low–ionic-strength nondenaturing polyacrylamide gel and visualized by autoradiography. Control reactions using a 200-fold molar excess of unlabeled oligonucleotide probes or a supershift assay were performed to demonstrate the specificity of the shifted DNA-protein complexes for NF-κB.

Transfection and Luciferase Assay

The luciferase reporter gene assay reflects NF-κB–dependent transcription. Briefly, endothelial cells were transfected with 2 μg pNF-κB-Luc plasmid (Stratagene, La Jolla, CA) by the lipofection method (Invitrogen, Carlsbad, CA). Four hours after transfection, cells were washed with phosphate-buffered saline (PBS) and incubated with M-199 (with 10% serum) for 24 hours. Then, the endothelial cells were stimulated with 90 μmol/L fatty acid (18:0 or 18:2) for 24 hours. Luciferase activity was determined following the instructions described in the luciferase assay kit (Promega, Madison, WI) using a luminometer.

IL-6 Production

After exposure to fatty acids and TNF, the media were removed from the wells and frozen immediately at −80°C until IL-6 analysis. The remaining cells were trypsinized and washed with PBS twice and resuspended in 0.2% sodium dodecyl sulfate with 0.2 mol/L NaOH for protein analysis.[21] IL-6 production and release into the medium was determined using the murine hybridoma cell line B9 (kindly supplied by Dr L.A. Aarden, Emeryville, CA) as described by Helle et al.[22] The B9 cell line viability is IL-6–dependent, and thus, the incorporation of ^{3}H-thymidine by viable cells is a reflection of the quantity of IL-6 produced by endothelial cells.

Endothelial Barrier Function (albumin transfer studies)

Endothelial barrier function was measured as transendothelial albumin transfer using polystyrene chambers with a 0.8-μm pore size polycarbonate membrane (Millipore, Bedford, MA) as described previously.[15] After achieving approximate confluence, endothelial monolayers were exposed to control or experimental media for 24 hours. Following treatments, chambers with endothelial cells attached to the membranes were washed with M-199 and exposed to 200 μmol/L bovine serum albumin (fatty acid–free; Sigma Chemical, St Louis, MO) in M-199 for 1 hour. After incubation, the albumin transferred across endothelial monolayers was determined using bromcresol green (Sigma) and recorded spectrophotometrically at 630 nm.

Lipid Analysis

Measurement of arachidonic acid release. Endothelial cells were cultured in M-199 enriched with 10% FBS and incubated with ^{3}H-arachidonic acid (0.2 mCi/mL medium) for 24 hours. Following incubation with radiolabeled 20:4, the cells were washed with serum-free M-199 medium and medium supplemented with 0.2% fatty acid–free BSA and then exposed to different 18-carbon fatty acids (90 μmol/L) for 6 hours. Subsequently, the media were collected and centrifuged at 3,000 rpm for 10 minutes to remove floating cells, and radioactivity was measured in the supernatant. The cells were immediately scraped in PBS, and lipids were extracted with chloroform: methanol (2:1) using a modified method of Takenaka et al.[23]

Separation of arachidonic acid and phospholipid fractions. Lipid extracts from each treatment were applied to a silica gel thin-layer chromatography (TLC) plate, and the separation of arachidonic acid and different phospholipids was performed using chloroform:methanol: ammonia (65:25:4) as a mobile phase. After identification of lipids in iodine vapor, arachidonic acid and phospholipid spots were scraped from the plate into scintillation vials with 10 mL scintillation cocktail (3a70B). The radioactivity of the samples was measured in a Tri-Carb2100TR liquid scintillation analyzer (Packard Instrument, Meriden, CT).

Statistical Analysis

Data were analyzed statistically using a 1-way ANOVA. For each endpoint, the treatment means were compared in pairs using the

Bonferroni procedure.[24] A P value of .05 or less was considered significant.

RESULTS

The effects of the 18-carbon fatty acids on cellular redox status were determined by measurement of cellular glutathione levels. Figure 1 demonstrates that both 18:0 and 18:2 significantly decreased glutathione levels. Compared with control cultures, treatment with 18:1 increased total glutathione, whereas 18:3 had no effect on intracellular glutathione levels.

The evidence suggests that oxidative stress can affect cellular metabolism by an increased expression of genes regulated by NF-κB. Interestingly, 18:0, the only saturated fatty acid, and 18:2 activated the transcription factor NF-κB most markedly (Fig 2), whereas 18:1 exposure to endothelial cells had little effect on the activation of this transcription factor. To test whether vitamin E can protect against fatty acid–induced activation of NF-κB, endothelial cells were pretreated with vitamin E for 24 hours before coexposure to fatty acids for an additional 6 hours (Fig 3). Vitamin E markedly decreased NF-κB binding induced by 18:0 or 18:2.

To determine whether 18:0- or 18:2-activated NF-κB can induce gene expression, endothelial cells were transfected with a plasmid (pNF-κB-Luc) encoding the bacterial protein luciferase. The expression of this construct is controlled by a promoter responsive to NF-κB. Results of the luciferase reporter gene assay are shown in Fig 4. Both 18:0- and 18:2-mediated activation of NF-κB were sufficient to induce NF-κB–dependent transcription in cultured endothelial cells. Compared with control cultures, luciferase activity was significantly higher in both 18:0- and 18:2-treated cells.

Figure 5 shows the effect of cellular incubation with control medium and media enriched with 18-carbon fatty acids on endothelial barrier function. Compared with control cultures, all fatty acids except 18:1 and 18:3 increased albumin transfer across endothelial monolayers. However, treatment with 18:2 disrupted endothelial barrier function most markedly.

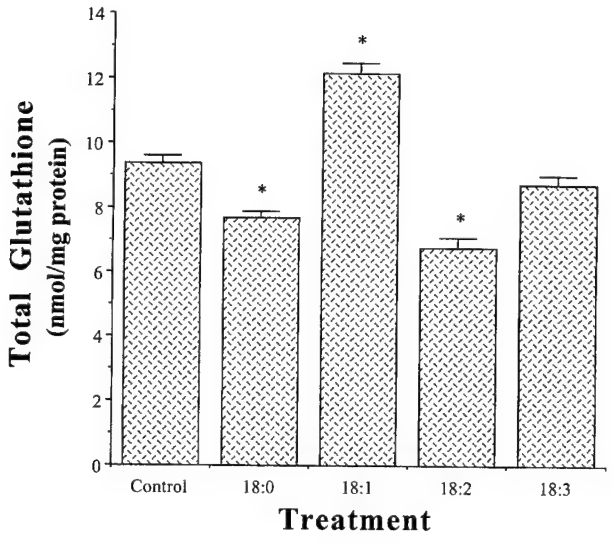

Fig 1. Effect of treatment with different 18-carbon fatty acids (90 μmol/L) on total glutathione levels in cultured endothelial cells. Cells were exposed to experimental media for 6 hours. Values are the mean ± SEM (n = 3). *Significantly different *v* control cultures.

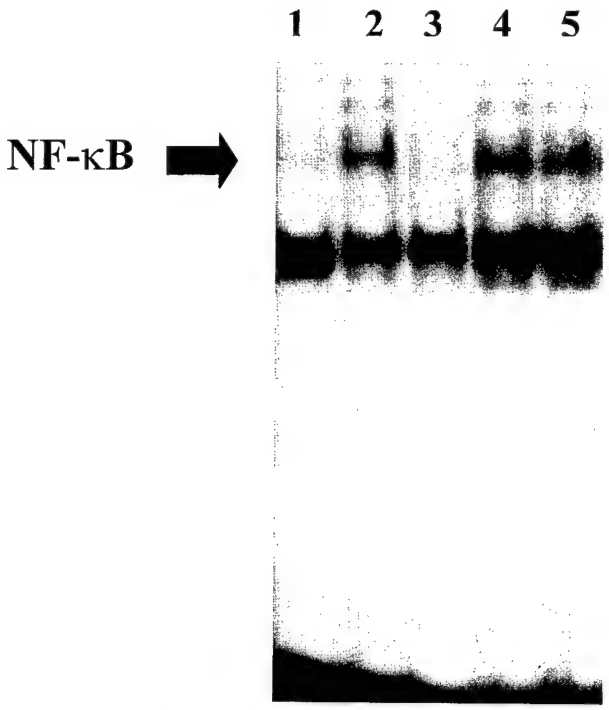

Fig 2. Effect of different 18-carbon fatty acids on activation of NF-κB. Cells were treated with the different fatty acids (90 μmol/L) for 6 hours. Lane 1, control; lane 2, stearic acid (18:0); lane 3, oleic acid (18:1); lane 4, linoleic acid (18:2); lane 5, linolenic acid (18:3). The specific binding of NF-κB was confirmed by both competitive (excess unlabeled oligonucleotide) and supershift assays.

Figure 6 shows IL-6 production in endothelial cells during fatty acid treatment for 9 hours followed by TNF exposure for an additional 3 hours. These data show that the cellular lipid environment can modify TNF-mediated inflammatory properties by selectively promoting endothelial cell–mediated production of IL-6. Compared with TNF treatment alone, preenrichment of endothelial cells with 18:2 followed by exposure to TNF resulted in a 100% increase in IL-6 production. In contrast, cellular preenrichment with 18:0, 18:1, and 18:3 had no further effect on the TNF-α–mediated production of IL-6.

The fatty acid–mediated changes in oxidative stress and other observed mediators of endothelial cell activation may be due to an increase in phospholipase A_2 activity and thus an increase in available arachidonic acid (20:4n-6) for metabolic activity. To test this hypothesis, cells were preenriched with radiolabeled 20:4 for 24 hours, carefully washed, and then treated with the 18-carbon fatty acids for an additional 6 hours. The surrounding media then were tested for cellular release of radiolabeled 20:4 (Fig 7). Cells were also harvested and analyzed for radioactivity in various lipid fractions, including phospholipids (Fig 8). Compared with control cultures (cells not enriched with 18-carbon fatty acids), cellular release of radiolabeled 20:4 was markedly increased only by cell exposure to 18:2 or 18:3 (Fig 7). Neither 18:0 nor 18:1 affected 20:4 release. Preenriching cultures with vitamin E decreased the fatty acid–mediated release of 20:4 into the media in all cultures independently of

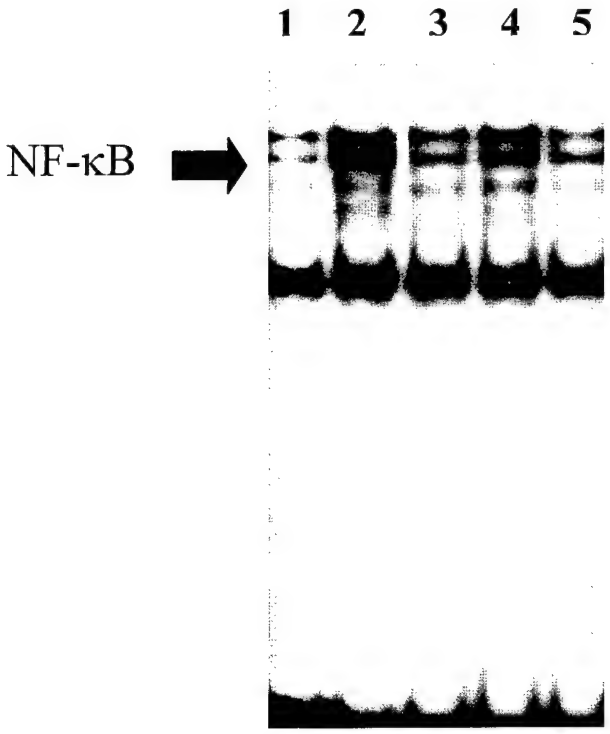

Fig 3. Effect of preenrichment with vitamin E on fatty acid–mediated activation of NF-κB. All cells were exposed to the different fatty acids (90 μmol/L) for 6 hours, and some cultures were first preenriched with vitamin E for 24 hours. Lane 1, control + vitamin E; lane 2, stearic acid (18:0); lane 3, 18:0 + vitamin E; lane 4, linoleic acid (18:2); lane 5, 18:2 + vitamin E.

the type of 18-carbon fatty acid to which the endothelial cells were exposed (data not shown). When analyzing for radiolabeled 20:4 in several types of cellular phospholipids, only its level in the phosphatidylethanolamine fraction was affected to a significant extent by 18-carbon fatty acid treatment. The most marked decrease in 20:4 incorporation into this phospholipid fraction was in cultures treated with 18:2, followed by cultures treated with 18:3. Neither 18:0 nor 18:1 treatment affected the 20:4 content in the phosphatidylethanolamine fraction. Thus, it appears that treatment with 18:2 or 18:3 can stimulate the release of 20:4 specifically from phosphatidylethanolamine.

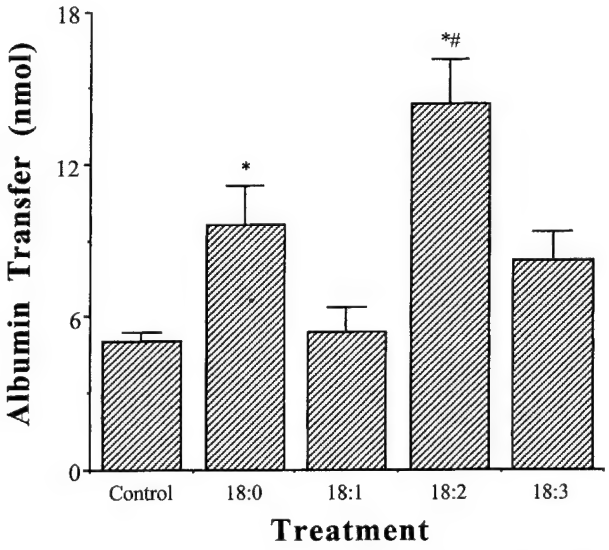

Fig 5. Effect of fatty acid exposure on albumin transfer across endothelial monolayers. Cultures were exposed to different 18-carbon fatty acids (90 μmol/L) for 24 hours. Subsequently, albumin transfer was measured over a 1-hour period. Values are the mean ± SEM (n = 6). *Significantly higher *v* control cultures. #Significantly higher *v* cultures treated with 18:0.

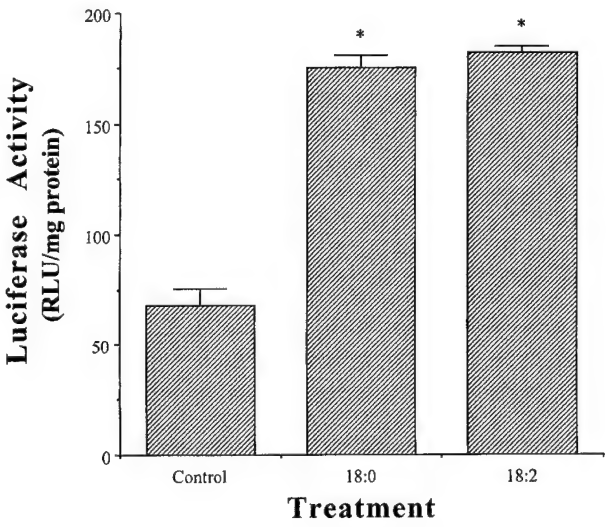

Fig 4. Effect of a 24-hour exposure to 18:0 or 18:2 on NF-κB–dependent transcription as measured by luciferase reporter gene assay. Data are expressed as relative light units (RLU) per mg protein. Values are the mean ± SEM (n = 3). *Significantly different *v* control cultures.

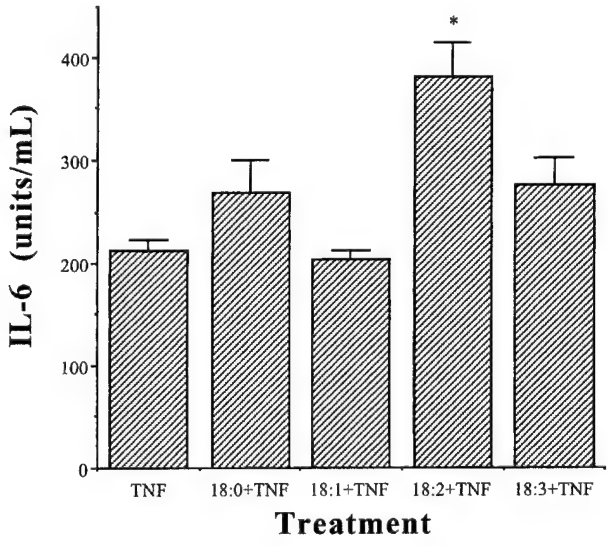

Fig 6. IL-6 production in endothelial cells after exposure to different 18-carbon fatty acids. Endothelial cells were treated with the different fatty acids (90 μmol/L) for 9 hours and with added TNF-α (500 U/mL) for an additional 3 hours. Values are the mean ± SEM (n = 3). *Significantly higher *v* control cultures.

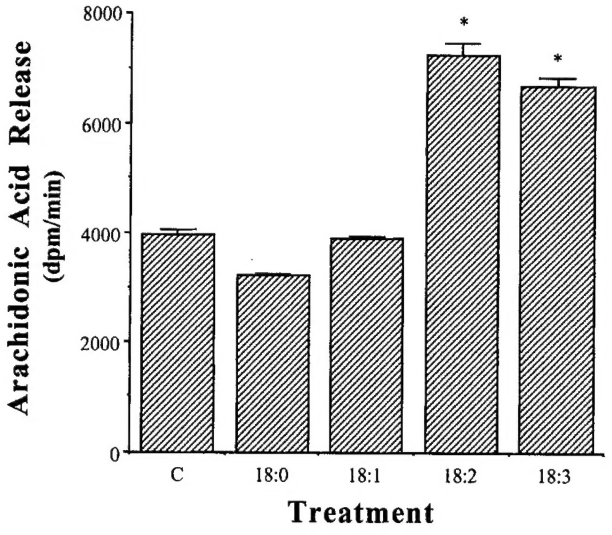

Fig 7. Release of radiolabeled 20:4 from endothelial cells following exposure to 18-carbon fatty acids (90 μmol/L) for 6 hours (cells were labeled with ^{3}H-arachidonic acid for 24 hours prior to fatty acid exposure). The media were collected and radioactivity was counted and expressed as dpm/min. Values are the mean ± SEM (n = 3). *Significantly higher *v* control cultures.

DISCUSSION

Although the mortality from coronary heart disease has declined recently, atherosclerosis and related vascular disorders still are the leading cause of death in the United States and other Western countries. Injury to or abnormal mechanisms of the vascular endothelium may be initiating events in the etiology of atherosclerosis. Dietary fat affects plasma lipids and lipoproteins and thus is linked to atherosclerosis.[25] The question then arises as to whether dietary saturated fats should be replaced by unsaturated fats. Unsaturated fats, especially monounsaturated[26,27] and n-3 or omega-3[28,29] fatty acids, may be beneficial

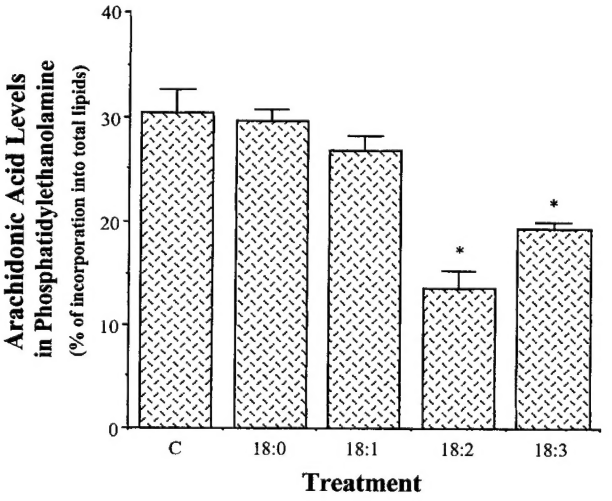

Fig 8. Incorporation of radiolabeled 20:4 into phosphatidylethanolamine. The experimental setup was the same as described in Fig 7. Lipids were obtained from total cell extracts and separated by TLC. Radioactivity was counted and expressed as dpm/min. Values are the mean ± SEM (n = 3). *Significantly lower *v* control cultures.

to human health. However, replacing saturated lipids with unsaturated and especially polyunsaturated lipids may not be desirable because of their ability to oxidize easily. The evidence supports the hypothesis that low-density lipoprotein undergoes oxidative modifications that increase its uptake by macrophages.[11] In fact, data from subjects with varying degrees of coronary atherosclerosis support the hypothesis that high serum polyunsaturated fatty acid levels, when insufficiently protected by antioxidants (eg, vitamin E), may indicate a higher risk of atherosclerosis.[30]

High levels of circulating triglyceride-rich lipoproteins (chylomicrons and very–low-density lipoprotein [VLDL]) have been implicated in the injury process of the endothelium.[31,32] Plasma chylomicron levels are elevated in humans after consuming a high-fat meal, and hepatic synthesis of VLDL is increased when the caloric intake is in excess of body needs. The hydrolysis of triglyceride-rich lipoproteins mediated by lipoprotein lipase, a key enzyme in lipoprotein metabolism that is associated with the luminal site of endothelial cells, may be an important source of high concentrations of fatty acid anions in the proximity to the endothelium.[33] It has been hypothesized that high levels of diet-derived fatty acids can cause endothelial injury or dysfunction and thus disrupt the ability of the endothelium to function as a selective barrier.[33,34] This would result in lipid deposition by allowing increased penetration of cholesterol-rich remnant lipoproteins into the arterial wall. In fact, the activity of lipoprotein lipase is increased in atherosclerotic lesions.[35,36] A recent report also provides evidence that lipoprotein lipase may be a chemoattractant for activated macrophages.[37] Lipoprotein lipase–derived remnants of lipoproteins isolated from hypertriglyceridemic subjects, as well as selective unsaturated fatty acids such as linoleic acid, were demonstrated to disrupt endothelial integrity.[38,39] In fact, a recent study has provided the first evidence that the lipolytic remnant products of triglyceride-rich lipoproteins produced after a meal rich in polyunsaturated fat are more injurious to arterial wall cells than those produced after a meal rich in saturated fat.[40] Furthermore, activated lipoprotein lipase induces TNF gene expression in macrophages and TNF production by this type of cell.[41] Thus, endothelial cells may be simultaneously exposed to free fatty acids and TNF.

As mentioned before, there is evidence that selected fatty acids, derived from the hydrolysis of triglyceride-rich lipoproteins, may be atherogenic by causing endothelial injury or dysfunction and subsequent endothelial barrier dysfunction.[42] In support of this hypothesis, we again confirm in the present study that, compared with all 18-carbon fatty acids, 18:2 disrupted endothelial barrier function most markedly. These findings agree with our earlier findings that when comparing fatty acid extracts derived from different animal fats and plant oils, the fat-induced disruption of endothelial barrier function was related to the amount of 18:2 present in the fat source.[43] These data suggest that among different fatty acids, linoleic acid may play a critical role in the pathogenesis of atherosclerosis.[44] This hypothesis is supported by the fact that adipose tissue levels of 18:2, which reflect the intake of this fatty acid over time, were positively associated with the degree of coronary artery disease.[45] In addition, concentrations of 18:2 were increased in the phospholipid fractions of human coronary

arteries in cases of sudden cardiac death due to ischemic heart disease.[46]

Several mechanisms were proposed to explain the injurious effects of 18:2 to endothelial cells. Due to the very low basal activity of endothelial cell elongases and delta 5 and delta 9 desaturases, arachidonic acid is not produced from 18:2 significantly in this type of cell.[47,48] Consequently, 18:2 accumulates within endothelial cells.[47,49] Moreover, 18:2 decreases the level of intracellular ATP[50] and proteoglycans,[51] enhances elastase-like activity,[52] and can yield nitrated oxidation species by reacting with nitric oxide–derived products.[53] The 18:2-mediated disruption of endothelial barrier function also may be caused by its ability to inhibit gap-junctional intracellular communication[54,55] and to induce intracellular oxidative stress.[40] Furthermore, 18:2, but not 18:0, can activate phospholipase A_2, as measured by the cellular release of 20:4 in neutrophils.[56] In fact, polyunsaturated free fatty acids that are liberated by phospholipase A_2 increased the formation of bioactive phospholipids in LDL, which stimulated endothelial cell activation and monocyte–endothelial cell interactions.[57]

In recent years, the role of oxidative stress has gained much attention in studies of lipid- and/or inflammatory cytokine–mediated endothelial cell dysfunction or injury. It is now generally accepted that LDL oxidation plays one of the most critical roles in atherogenesis. LDL can be oxidized in the subendothelial space, which lacks many of the antioxidants present in the whole blood. Furthermore, dietary oxidized lipids can be absorbed by the small intestine, be incorporated into chylomicrons, appear in the bloodstream, and thus contribute to the total body pool of oxidized lipids.[58] Including oxidized corn oil (a rich source of 18:2) in a diet accelerated the development of fatty streaks in cholesterol-fed rabbits,[59] suggesting that the consumption of oxidized lipids (eg, high–corn oil diets) may be an important risk factor for atherosclerosis. Our data support the notion that omega-6 fatty acids, and especially fats rich in 18:2, are atherogenic by activating vascular endothelial cells and by promoting an inflammatory response. We clearly show that 18:2 most markedly amplifies TNF-mediated IL-6 production by endothelial cells. An increase in oxidative stress and subsequent activation of NF-κB may be one of the main mechanisms of the inflammatory properties of 18:2. However, there appears to be no relationship between the degree of unsaturation of fatty acids and endothelial cell activation. In fact, stearic acid (18:0) appears to activate endothelial cells more markedly than either 18:1 or 18:3. Furthermore, 18:1 had little or no effect on endothelial cell activation. Interestingly, when studying lipoproteins from subjects consuming different types of dietary fat, eg, oleic acid or linoleic acid, only the percentage of 18:2 in LDL correlated strongly with the extent of oxidizability and macrophage degradation of these lipoproteins.[60]

It is not clear why 18:0 decreased cellular glutathione and increased NF-κB activation so markedly. Although 18:0, as a saturated fatty acid, does not undergo peroxidative modifications, it may induce perturbations in cellular metabolism, which secondarily can result in oxidative stress and be responsible for the observed decreases in glutathione concentrations. On the other hand, 18:0 may influence gene expression or signal transduction pathways that are more substantial than its unknown or secondary effects on oxidative stress. The fact that preenrichment of cultures with vitamin E can block the activation of NF-κB suggests that this fatty acid can modify the cellular lipid milieu, leading to an imbalance in oxidative stress/antioxidant status and to endothelial cell activation. Because of its lack of double bonds, 18:0 may affect the membrane properties of endothelial cells differently compared with fatty acids with *cis* double bonds. 18:0 also may be taken up and metabolized differently than fatty acids that contain double bonds. In fact, once taken up by endothelial cells, 18:0 is randomly distributed among membrane phospholipids,[61] whereas unsaturated fatty acids are initially preferentially incorporated into phosphatidylcholine and then can undergo a time-dependent transfer to phosphatidylethanolamine.[61] Furthermore, using electron-spin resonance studies, we found that of all 18-carbon fatty acids, only 18:0 increased membrane fluidity.[62] In that same study, a relationship between membrane fluidity and fatty acid compositional alterations in cellular phospholipids was observed, ie, only the unsaturated fatty acids, not 18:0, decreased the cellular arachidonic acid content. These and our present data suggest that 18:0 may have unique membrane-modifying effects.

In summary, our data suggest that omega-6 fatty acids appear to be most effective in activating endothelial cells and in contributing to an inflammatory response. In contrast, 18:1 does not appear to activate endothelial cells, and in fact may protect endothelial cells against oxidative insult.[63] These data support the concept that the substitution of dietary monounsaturated fatty acids and not polyunsaturated fatty acids for saturated fatty acids might be preferable for the prevention of cardiovascular disease.

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